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CONTENTS

No. 1. February, 1948

PAGE

Lynn W. Gardner
The effects of thiourea and phenylthiourea upon the development of Eleutherodactylus ricordii 1

Cheney, Ralph Holt
Caffeine effects on fertilization and development in Arbacia punctulata . 16

Redfield, Alfred C., and Charles M. Weiss
The resistance of metallic silver to marine fouling 25

Wilber, Charles G., and Gerald R. Seaman
The lipids in Colpidium campylum 29

Smith, F. G. Walton
Surface illumination and barnacle attachment 33

Klotz, I. M., A. H. Schlesinger, and F. Tietze
Comparison of the binding ability of hemocyanin and serum albumin for organic ions 40

Marvel, Rosemary Martin, and Kenneth C. Fisher
Developmental changes in the viability of squid embryos after subjection to cyanide 45

Freeman, John A., and Karl M. Wilbur
Carbonic anhydrase in molluscs 55

Williams, Carroll M.
Physiology of insect diapause. III. The prothoracic glands in the Cecropia silkworm, with special reference to their significance in embryonic and postembryonic development 60

Fry, F. E. J., and J. S. Hart
The relation of temperature to oxygen consumption in the goldfish 66

Program and abstracts of seminar papers presented at the Marine Biological Laboratory, Summer of 1947 (Addendum) 78

No. 2. April, 1948

Shapiro, Herbert
Retardation of cell division by vitamin C in physiological concentrations 79

Beers, C. Dale
Excystment in the ciliate Bursaria truncatella 86

Beers, C. Dale
The ciliates of Strongylocentrotus drobachiensis: incidence, distribution in the host, and division 99

WICHTERMAN, RALPH
The biological effects of x-rays on mating types and conjugation of Paramecium bursaria 113
ROGICK, MARY D.
Studies on marine bryozoa. II. Barentsia laxa Kirkpatrick 1890 128
MILLER, MILTON A., J. C. RAPEAN, AND W. FOREST WHEDON
The role of slime film in the attachment of fouling organisms 143
COE, WESLEY R.
Nutrition and sexuality in protandric gastropods of the genus Crepidula 158

No. 3. JUNE, 1948

GREGG, JAMES H.
Replication of substrate detail by barnacles and some other marine organisms 161
BERKELEY, EDMUND
Spindle development and behavior in the giant amoeba 169
BEVELANDER, GERRIT, AND PAUL BENZER
Calcification in marine molluscs 176
GOLDIN, ABRAHAM
Regeneration in Perophora viridis 184
MENDES, MARTA VANNUCCI
Histology of the corpora allata of Melanoplus differentialis (orthoptera: Saltatoria) 194
MITTLER, SIDNEY
Influence of genetic environment on the reduction of bristles by the dichaete gene in Drosophila melanogaster 208
RICHARDS, A. GLENN, AND FRANCIS H. KORDA
Studies on arthropod cuticle. II. Electron microscope studies of extracted cuticle 212
WEISS, CHARLES M.
Seasonal and annual variations in the attachment and survival of barnacle cyprids 236
KING, JOSEPH E.
A study of the reproductive organs of the common marine shrimp, Penaeus setiferus (Linnaeus) 244
EDMONDSON, W. T.
Rotatoria from Penikese Island, Massachusetts, with a description of Ptygura agassizi n. sp. 263
BARRON, E. S. GUZMAN, J. E. SEEGMILLER, E. G. MENDES, AND H. T. NARAHARA
The effect of nitrogen mustards on the respiration and fertilization of sea urchin sperm and eggs 267
MAKINO, SAJIRO
A study of the chromosomes in two species of bats (Chiroptera) 275

3
28
43
58
61
69
76
84
94
08
12
36
44
263
267
275

Vol. 94, No. 1 February, 1948

THE
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THE EFFECTS OF THIOUREA AND PHENYLTHIOUREA UPON THE DEVELOPMENT OF ELEUTHERODACTYLUS RICORDII

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The leptodactylid toads of the genus *Eleutherodactylus* are unusual among anurans in that they lay large-yolked, unpigmented, terrestrial eggs and have no aquatic larval stage. The embryology of one member of this group, the Jamaican species *E. nubicola,* has been described in some detail (Lynn, 1942) and it was pointed out that during the development within the egg membranes certain characters which are found in ordinary aquatic tadpoles appear very transiently while others are never present at all. Thus, for example, the embryo possesses a broad vascular tail which is large and well developed during the latter part of intraoval life but degenerates and disappears near the time of hatching. Neither external nor internal gills are ever formed, there is no true opercular cavity and the ventral sucker and horny teeth of the typical tadpole are entirely lacking. The fore and hind limbs appear simultaneously and grow steadily throughout the embryonic period and at the time of hatching the young emerge as fully developed little frogs.

It is well known that in ordinary anurans such as *Rana pipiens* the metamorphosis of the tadpole into the frog is brought about through the agency of the thyroid gland which, late in larval life, begins a period of intense secretory activity resulting in a heightened level of thyroid hormone in the blood stream. The loss of gills and tail, shortening of the intestine, rapid growth and differentiation of the limbs, shedding of the chitinous beak, appearance of the tympanum and many other features of metamorphosis have been shown to be directly dependent upon this gradually increasing level of thyroid hormone. Thyroidectomized tadpoles remain in the larval state indefinitely unless thyroxin or iodine is administered to them. On the other hand, if normal tadpoles are treated with thyroxin at an early age they can be made to metamorphose precociously, producing tiny, but perfectly formed young frogs.

In view of these facts it seems possible that the telescoping of the larval stages in the development of *Eleutherodactylus* might be regarded as an exaggeratedly precocious metamorphosis resulting from an unusually early and intense functioning of the thyroid in this genus. The author has earlier presented some evidence, based on the histology of the thyroid at different embryonic stages, indicating that the gland is indeed precociously activated in this animal (Lynn, 1936). More recently Brink (1939) has made a study of the histology and cytology of the thyroid in *Arthroleptella bicolor villiersi,* a South African ranid with a somewhat similarly

abbreviated larval history, and has concluded that in this form also, the release of the thyroid hormone into the blood stream occurs at an unusually early stage.

It is clear that this evidence based upon the histological picture presented by the thyroid must be supplemented by experimental evidence before it can be regarded as conclusive. In the summer of 1941 the present author, working in Jamaica, B. W. I., attempted an experimental approach by removal of the pituitary anlage in the early embryo of *E. nubicola*. This operation, since it results in absence of the thyrotrophic hormone of the pituitary, prevents normal differentiation and functioning of the thyroid gland and thus provides a method for ascertaining the normal role of the thyroid in the developmental processes under investigation. Unfortunately these experiments were inconclusive because of the difficulty encountered in keeping the animals alive after the operation. Although the operation was relatively simple and involved removal of only a small bit of tissue, the embryos did not heal with the readiness exhibited by most amphibian larvae and despite repeated attempts, no operated animals survivied more than a few days.

With the recent development of various thyroid-inhibiting drugs, thiourea and related compounds (Kennedy, 1942; Richter and Clisby, 1942) and a number of the sulfonamides (Mackenzie, Mackenzie and McCollum, 1941; Mackenzie and Mackenzie, 1942, 1943; Astwood, 1943; Astwood, Bissell and Hughes, 1945), a new approach to the problem is possible since administration of these drugs will effectively block the production of thyroid hormone without the necessity of any surgical treatment.

The present paper is a report of such experiments carried out on the embryos of *Eleutherodactylus ricordii planirostris* (Cope), a species which is native to Cuba but has now, through accidental introductions, become well established in many parts of Florida.

Materials and Methods

The eggs used for this study were collected at Gainesville, Florida between August 19 and September 2, 1946.[1] The breeding habits of *E. ricordii* in Florida have been described by Deckert (1921), Skermer (1939) and Carr (1940). The eggs are laid in moist situations under loose boards or stones and the female remains with the eggs until they hatch. Goin (1947), in a study made at Gainesville, found that the average number of eggs per clutch is 16 and that the average period of development within the egg membranes is 15.6 days. For the present work 15 clutches of eggs were used. Each clutch was designated with a letter, the 15 groups thus constituting Series A to O. The total number of eggs available was 193 and the number per clutch ranged from 5 to 21.

Although these eggs are normally terrestrial, experience with the Jamaican species had shown that they can survive when immersed in water. It was therefore possible to administer the drugs simply by raising the eggs in the solutions, thus avoiding the necessity of injection. In each experiment a few eggs were kept on moist sand in a small flower-pot covered with a glass plate in order to simulate na-

[1] The author is indebted to Prof. J. Speed Rogers, Head of the Department of Biology, The University of Florida, for laboratory facilities during the course of the experiments and to Dr. C. J. Goin for suggestions and assistance in the collecting of specimens. Sectioning and study of the material was carried out at The Catholic University of America and at The Marine Biological Laboratory, Woods Hole, Massachusetts.

tural conditions; others were raised in tap-water and still others were raised in tap-water containing the appropriate concentration of the drug to be tested.

All of the eggs in a single batch are at the same stage of development at any given time but, of course, those of different batches were at various stages when collected. For this study only those series were used which were collected at the neural plate stage or earlier. All eggs were kept in the laboratory until they reached the early limb-bud stage before any treatment was instituted. This procedure was adopted because it insured that all embryos received treatment for comparable periods and also because previous experience had shown that successful removal of the jelly coats and vitelline membrane is difficult at earlier stages. At the limb-bud stage the thyroid gland is not yet differentiated (Lynn, 1936, 1942) so that in all of these experiments the treatment with thyroid-inhibiting drugs was instituted before the beginning of thyroid function.

Removal of the egg membranes was carried out in sterile Holtfreter's solution by means of finely ground watchmaker's forceps.

Daily observations were made under the binocular microscope and at various intervals embryos were fixed for sectioning. Fixation was in a 1:1 mixture of Bouin's fluid and cellosolve. The embryos were later dehydrated in cellosolve, cleared in xylol, sectioned at 10 μ and stained with Mallory's triple stain.

Results

1. Effect of the egg membranes upon the developmental rate of eggs raised in water.

Although eggs placed directly into tap-water were found to survive and to develop into froglets of normal appearance it became obvious early in the course of the work that such eggs were markedly retarded in their rate of development as compared with those kept in air on moist sand. On the other hand, if the jelly layers and vitelline membranes were removed from the eggs to be kept in fluid, then the developmental rate closely paralleled that of eggs kept on sand with all their coverings intact.

The details of a single experiment may be cited to illustrate this effect. The eggs of Series D were in early cleavage when collected on August 19. They were kept on moist sand for 5 days at which time all were well-developed embryos with both pairs of limbs present as buds, large vascular tails and lightly pigmented bodies (the "limb-bud stage"). At this time some of the eggs were dissected free of the surrounding jelly and membranes and placed in tap-water, others were placed in tap-water with all coverings intact and others were kept on moist sand. The eggs kept on sand developed normally and all hatched 10 days later (15th day of development). Eggs in tap-water without membranes paralleled those kept on sand. Differentiation and growth of limbs and digits, intensification of pigment, growth and later degeneration of the tail and other grossly visible changes occurred concomitantly in the two groups and at the time when the eggs kept in air were hatching, those kept in water were indistinguishable from them. Young frogs which were left in water after this time died within two days but those removed to moist sand lived normally. This is undoubtedly to be attributed to the change from cutaneous to pulmonary respiration which occurs at this time. During embryonic existence in the absence of any gills the respiration is cutaneous, probably mainly

through the thin-walled vascular tail. At late stages the tail begins to degenerate and it usually disappears within a day after hatching, when the lungs have come into function.

The eggs which were left in their membranes and raised in water showed a retardation almost immediately. Differentiation of the limbs was slow and the difference in pigmentation between these animals and the members of the control groups was particularly noticeable. By the 15th day of development, the time when the controls hatched, these embryos were at the stage which the controls had reached on the 8th day. They continued to develop slowly and reached what appeared to correspond to the hatching stage on about the 26th day. The embryos seemed weak however and none hatched spontaneously. When freed from the egg membranes by forceps they swam sluggishly, but when removed to moist sand they survived successfully.

The retarding effect of the egg membranes is shown in Figures 1 and 2 which are photographs of two individuals of Series H. These embryos were eleven days old when photographed and both had been kept in tap water from the 5th day of development. The embryo shown in Figure 2 had all coverings intact while that shown in Figure 1 had the membranes removed at the time of its immersion in water. The difference in the differentiation of the limbs and digits is particularly striking but sectioned material reveals that this is merely one aspect of a general retardation in developmental rate. It seems probable that this effect is due to a reduction of the rate of gas diffusion to and from the embryo, but no determinations of respiratory rate have been made to test this.

This effect having been demonstrated, all the experiments with thyroid-inhibiting drugs were carried out with eggs freed from the jelly layers and vitelline membrane.

2. Effects of thiourea treatment upon development.

A total of 71 eggs taken from 12 different batches was used for study of the effects of thiourea treatment. Three different concentrations were tested and, as previously noted, separate controls were run for each batch of eggs.

The lowest concentration of thiourea used was 0.001 per cent. Only 8 eggs, taken from Series A and B, were exposed to this concentration. The development of these did not seem to be affected in any way, the rates of growth and differentiation being the same as those of tap-water controls, and use of this concentration was therefore discontinued.

Twenty-four embryos from 5 different series of eggs were raised in 0.005 per cent thiourea. These animals showed no significant retardation in their rate of devel-

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PLATE I

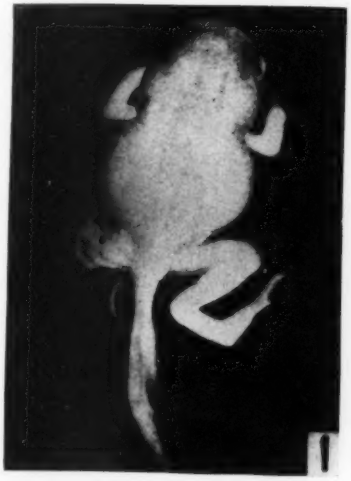
FIGURE 1. Specimen from Series H, removed from jelly layers and vitelline membrane and placed in tap-water on the fifth day of development. Preserved and photographed on the eleventh day. × 8.

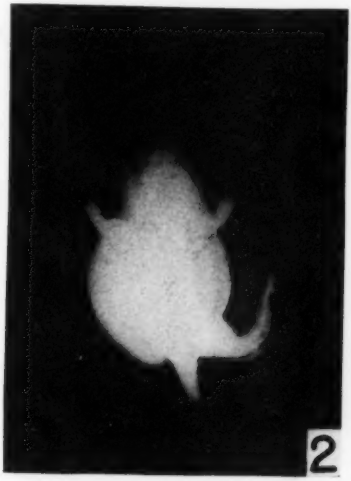
FIGURE 2. Specimen from Series H, raised in tap-water from the fifth to eleventh days with jelly layers and vitelline membrane intact. (Coverings removed before photographing.) × 8.

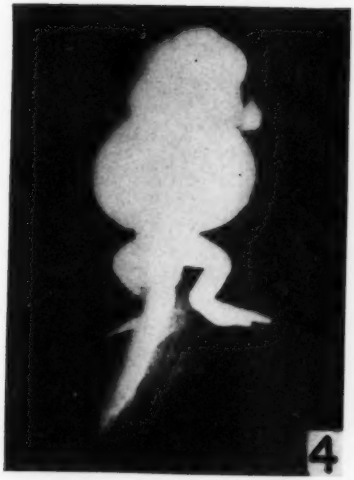
FIGURE 3. Specimen from Series H, raised in 0.005 per cent thiourea from the fifth to eleventh day of development. × 8.

FIGURE 4. Specimen from Series H, raised in 0.005 per cent phenylthiourea from the fifth to eleventh day of development. × 8.

PLATE I







opment but at the time when the tap-water controls were exhibiting marked degeneration of the tail, the tails of the experimental frogs remained large and vascular. This being the case, the latter animals were able to survive in water for an indefinite period while controls left in water invariably died when the tails had been reduced to small size. Because of the author's short stay in Florida, the longest period of survival of these experimental larvae beyond the time of death of the controls was 9 days. However, these animals were all active and healthy in appearance when fixed and there is no reason to suppose that they could not have lived much longer if some means of feeding them could have been found.

Figures 3 and 6 illustrate the effect of treatment with 0.005 per cent thiourea. The animal shown in Figure 3 is a specimen from Series H photographed at 11 days of age after 6 days in this solution. Comparison with the tap-water control of the same series (Fig. 1) shows that there is no significant difference between the two at this time ("pre-hatching stage"). Figure 6 shows an individual of Series N which was kept in 0.005 per cent thiourea for 11 days and a tap-water control of the same series is shown in Figure 5. It will be seen that the latter has passed the "hatching stage," having lost the tail and assumed the adult body form, while the experimental animal shows no signs of tail degeneration. Differentiation of the limbs in the two is, however, essentially the same.

Thirty-nine embryos from 7 different series of eggs were raised in 0.05 per cent thiourea solution. This concentration caused a definite retardation of development which first became apparent about 4 or 5 days after the beginning of treatment. From this time on, the experimental animals lagged behind the tap-water controls so that when the latter reached the "hatching stage," the former still had poorly developed digits, large vascular tails and ill-defined pigment patterns. Further development of these embryos was extremely slow and animals kept in 0.05 per cent thiourea for 10 days beyond the "hatching time" of the controls still exhibited several embryonic features in addition to the large larval tail. This is shown in Figures 7 and 8. These two embryos of Series C had been in fluid for 10 days. The control (Fig. 7) is very near the hatching stage with a much reduced tail and well-developed digits. The experimental animal (Fig. 8) has a tail of maximum size and shows considerable retardation of limb differentiation and pigment pattern development.

It is unfortunate that considerations of time made it impossible to carry these animals for longer periods beyond the "hatching time" for it appears probable that, in the case of embryos treated with 0.05 per cent thiourea, certain of the developmental features are not merely retarded but actually inhibted. Complete differentiation of the limbs was never attained and there was never any sign of metamorphic

PLATE II

FIGURE 5. Specimen from Series N, raised in tap-water from the fifth to the sixteenth day of development. × 8.

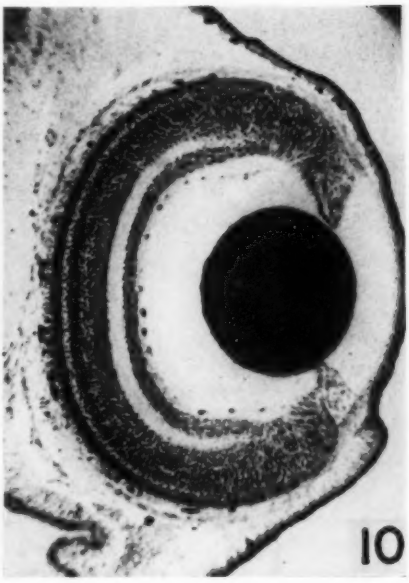
FIGURE 6. Specimen from Series N, raised in 0.005 per cent thiourea from the fifth to the sixteenth day of development. × 8.

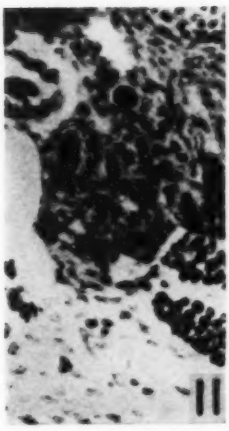
FIGURE 7. Specimen from Series C, raised in tap-water from the fifth to the fifteenth day of development. × 8.

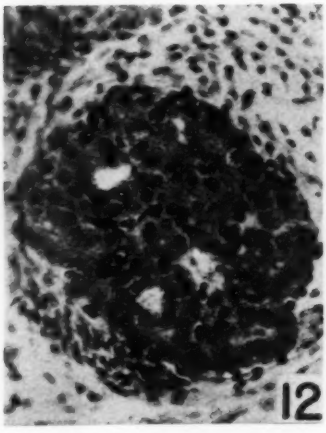
FIGURE 8. Specimen from Series C, raised in 0.05 per cent thiourea from the fifth to the fifteenth day of development. × 8.

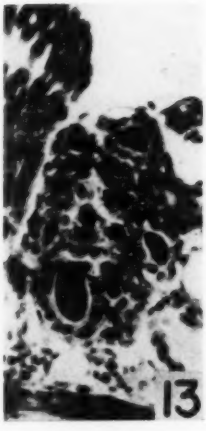
PLATE II

PLATE III









degeneration of the tail during the course of the experiment. These are clearly effects which would be expected to result from inhibition of the metamorphosis-inducing properties of the thyroid but, since the animals when fixed still retained considerable amounts of yolk, the possibility remains that the effect is attributable to a general retardation of metabolic rate. Raising the embryos in 0.05 per cent thiourea for much longer periods, until complete utilization of the yolk, would doubtless settle this point.

3. Effects of phenylthiourea treatment upon development.

Twenty eggs from 5 different series were treated with 0.005 per cent phenylthiourea. These all showed a retardation of development which was essentially the same as that caused by 0.05 per cent thiourea. Degeneration of the tail was prevented and complete differentiation of the limbs and digits was never attained.

An additional and very striking effect produced by phenylthiourea, however, was a rapid and complete loss of pigment. At the limb-bud stage, when treatment was instituted, the embryos had a light peppering of melanophores over the dorsal surface and the pigmented coat of the retina was quite black. In every case, however, the experimental larvae were noticeably lighter than the controls by the third day after the beginning of treatment, and by the fifth or sixth day all visible dark pigment had disappeared. The eyes became white and the skin took on a translucent golden appearance indicating decoloration of the melanophores but not of the lipophores. It was also noted that these embryos after about ten days in the solution gave evidence of an abnormally high blood pressure or increased strength of heart-beat, the head and fore-limbs moving rhythmically with each heart-beat. The heart rate was not significantly different from that of the controls however.

An illustration of the effect of 0.005 per cent phenylthiourea upon general development and pigmentation will be seen in Figure 4. The animal shown in this photograph is another individual of Series H which was kept in the phenylthiourea solution for six days after the limb-bud stage. Comparison with the tap-water control (Fig. 1) or the animal kept in 0.005 per cent thiourea (Fig. 3) shows the pronounced depigmentation and the decided retardation in differentiation of the limbs. The loss of pigment in the pigmented coat of the eye is best seen in sectioned material as shown in Figures 9 and 10 which are photographs of sections of the eyes of the same animals shown in Figures 1 and 4. The pigmented coat and iris of the phenylthiourea-treated animal (Fig. 10) is almost completely decolorized despite the fact that this animal had been exposed to the drug for only 6 days.

4. Effects of thiourea and phenylthiourea upon the histology of the thyroid gland.

PLATE III

FIGURE 9. Section through the eye of the control animal shown in Figure 1. × 150.

FIGURE 10. Section through the eye of the phenylthiourea-treated animal shown in Figure 4. × 150.

FIGURE 11. Central section through the thyroid gland of the control animal shown in Figure 1. × 300.

FIGURE 12. Central section through the thyroid gland of the phenylthiourea-treated animal shown in Figure 4. × 300.

FIGURE 13. Central section through the thyroid gland of the thiourea-treated animal shown in Figure 3. × 300.

Examination of serial sections of control animals and those subjected to the various treatments described above provides a close correlation between the grossly visible effects upon development and the histological changes induced in the thyroid.

The results of treatment with 0.005 per cent phenylthiourea may conveniently be described first. Sections of the thyroids of experimental and control embryos fixed on the third day of treatment (eighth day of development) already exhibit well-marked differences. The control thyroid consists of relatively few small, primary follicles with low cuboidal epithelium and with the lumina occupied by a homogeneous blue-staining colloid. It represents a fairly early stage and only a mild degree of thyroid activity. The glands of the treated animal are only slightly enlarged but the follicular epithelium is predominantly columnar, some vacuolation of the colloid has occurred and the vascularization of the thyroid has increased. By the sixth day of treatment the contrast is much more striking and this stage has been chosen for illustration. Figure 11 is a photomicrograph of a central section of the thyroid of a tap-water control from Series H at this time. It will be noted that the follicular epithelium is cuboidal to low columnar and that all follicles contain fairly large masses of homogeneous red-staining colloid. This is a relatively active, but not a hyperactive, gland. The thyroid of the treated embryo is shown in Figure 12. It is markedly enlarged and the follicular epithelium is hyperplasic. Mitotic figures are common, three of them being seen in this photograph. Most follicles are completely collapsed and those which are not contain almost no stainable colloid. The hyperemia is indicated by the numerous blood corpuscles scattered about between the follicles. Essentially this same picture of intense activity is seen in all later stages studied which include animals up to the twenty-fourth day of development (nineteenth day of treatment) and the later stages are therefore not illustrated. There is no evidence in this material of any regression in activity during the period studied but perhaps this would have been observed if treatment could have been continued for a longer time.

The thyroids of animals treated with 0.05 per cent thiourea present the same picture as that produced by 0.005 per cent phenylthiourea and therefore need not be discussed in detail. It will be remembered that both of these treatments caused the same retardation in development.

Thyroids of animals kept in 0.005 per cent thiourea differ from those of controls only in that they are slightly enlarged and show increased vascularity. There is no significant difference in the amount or nature of the colloid present or in the height of the follicular epithelium. The thyroid shown in Figure 13 is that of the animal shown in Figure 3 which had been in 0.005 per cent thiourea for 6 days. It may be compared with the control of the same age (Figure 11). Animals treated for longer periods show no more pronounced effects. Despite the slight histological change exhibited by the thyroid in this case, some change in the amount or nature of the hormone produced must be postulated since tail degeneration is definitely prevented by this concentration.

The available sectioned material of animals treated with 0.001 per cent thiourea shows no points of difference from the controls. This was to have been expected since this concentration produced no detectible effects on development.

Discussion

Although only a few studies of the effects of thyroid-inhibiting drugs upon amphibians have as yet been made it has been conclusively demonstrated that these substances can produce effects upon the larva which are comparable to those resulting from thyroidectomy. Gordon, Goldsmith and Charipper (1943, 1945) showed that *Rana pipiens* tadpoles kept in 0.033 per cent thiourea retain the larval tail, gills and mouth-parts and fail to attain complete differentiation of the limbs. In other words they do not metamorphose, although they do continue to grow and may reach excessive sizes. Metamorphosis usually occurs promply when treatment ceases, although it may be delayed if treatment has been of long duration. Similar effects produced by thiouracil in a concentration of 1:2000 have been reported for *Rana clamitans* (Hughes and Astwood, 1944) and for *Rana pipiens* (Lynn and Sister Alfred de Marie, 1946).

On the basis of the present work it is clear that in *Eleutherodactylus*, as in *Rana*, there are certain developmental features which are dependent upon thyroid stimulation and are inhibited when normal production of the thyroid hormone is interfered with. The most noteworthy of these features are the resorption of the larval tail and the completion of differentiation of the limbs and digits. On the other hand, however, these experiments show that many of the developmental processes which are under thyroid control in ordinary anurans are to a greater or less degree, independent of such control in *Eleutherodactylus*. Thus even under conditions of what seems to be complete thyroid inhibition (treatment with 0.05 per cent thiourea or 0.005 per cent phenylthiourea) no tadpole-like mouth-parts, operculum or gills are ever formed and the limbs do develop to a considerable degree before showing any inhibition. In other words the treatment with thyroid-inhibitors does not result in the appearance of any larval characters which are normally absent in the species but it does cause an indefinitely prolonged retention of certain features which are normally very transient.

In an earlier discussion of this matter (Lynn, 1936) the author has pointed out that the evolutionary change which resulted in the atypical life history of *Eleutherodactylus* could conceivably have been brought about through a relatively simple genetic change, namely one which resulted in a precocious activation and functioning of the endocrine complex governing metamorphosis. The telescoping of the larval stages and early assumption of the adult body form could result from this and the later stages of intra-oval development could then be properly regarded as a precocious metamorphosis. The present experiments indicate quite clearly that the evolution of terrestrial development in these frogs can not be reduced to such simple terms. The thyroid stimulus undoubtedly plays a part in some of the later differentiations but many features of the embryogeny are carried out independently of the thyroid and it is obvious that in this anuran the genetic constitution is such that many of the tissues are able to undergo complete differentiation to the adult form without the endocrine intervention which is so essential in most amphibians. The development of the *Eleutherodactylus* embryo can, therefore, be more accurately described as a "direct development" rather than a "prococious metamorphosis within the egg."

The depigmentation effect exhibited by phenylthiourea but not by thiourea merits some discussion. Even before the discovery of the goiterogenic properites of

505693

phenylthiourea it was reported by Richter and Clisby (1941) that continued administration of this substance to black rats causes graying of the hair, and also that cessation of treatment is followed by return of pigment. This phenomenon has been further studied by Dieke (1947) who finds also that alpha-naphthyl thiourea causes depigmentation of the skin of the rat. Neither of these drugs, in the doses used, had any effect upon the eye pigment of the rat.

Juhn (1944, 1946) has reported an effect of thiouracil administration upon feather pigmentation in the Brown Leghorn fowl but this seems to be attributable to the thyroid inhibition rather than to any direct influence upon the pigment cells for the modifications produced are the same as those which result from thyroidectomy.

Only one published account is concerned with the effects of thiourea derivatives upon the pigmentation of amphibians. This report (Lynn and Sister Alfred de Marie, 1946) records a reversible blanching observed in tadpoles of *Rana pipiens* raised in 0.05 per cent thiouracil. Further experiments in this laboratory have revealed that a definite depigmentation of the skin of *Rana pipiens* tadpoles is also produced by treatment with allylthiourea, phenylthiourea, amenobenzoic acid and sulfanilamide. In none of these cases, however, was the pigmentation of the eyes lost. The *Eleutherodactylus* embryo thus seems to be particularly susceptible to this action, showing much more complete and rapid depigmentation than does *Rana pipiens*.

It is noteworthy that a depigmentation of the skin and eyes very similar to that caused by phenylthiourea was reported much earlier (Lewis, 1932) in *Rana sylvatica* larvae treated with certain of the indophenol dyes. Moreover Figge (1938a), testing the effectiveness of these dyes upon various amphibian larvae, found a marked difference in sensitivity in different species. Larvae of *Necturus* were very readily depigmented in relatively low concentrations of the dyes; larvae of *Rana sylvatica* were somewhat less sensitive; larvae of *Amblystoma mexicanum* and *Rana catesbiana* were still less readily affected and *Amblystoma punctatum* larvae were least sensitive of all. Figge points out that this order of sensitivity parallels the order of metabolic rate of the different animals studied. *Necturus*, the most sensitive, has the lowest metabolic rate; *A. punctatum*, the least sensitive, has the highest; while *A. mexicanum* is intermediate in both respects. It is unfortunate that, because of the lack of any studies upon the metabolism of the *Eleutherodactylus* embryo, no conclusions concerning a possible relation between metabolic rate and sensitivity to the depigmentation effects of phenylthiourea can be drawn.

The basis for the depigmentation effect of the indophenol dyes has been investigated by Figge (1938b, 1939, 1940, 1941) who finds that phenol indophenol does not destroy pigment granules once formed but does prevent further formation of granules by affecting the enzyme system responsible for pigment production. Specifically, it was found that the dye inhibits the enzyme tyrosinase and thus prevents the production of melanin by the action of tyrosinase on tyrosine. The mechanism of the inhibition is apparently to be found in the fact that the dye shifts the oxidation-reduction potential of the substrate away from the optimum potential for tyrosinase activity. Presumably any substance which would cause such a shift in substrate potential would be equally effective in inhibiting pigment formation.

In view of these findings for the indophenol dyes, it is not surprising that recent studies indicate that the depigmentation effect of phenylthiourea and others of the thiourea derivatives are also to be attributed to tyrosinase inhibition although

whether the precise mechanism of inhibition is the same as that which seems to be obtained in the case of the dyes has not as yet been ascertained. Bernheim and Bernheim (1942) demonstrated inhibition of tyrosinase in vitro by phenylthiourea (phenylthiocarbamide), Paschkis, Cantarow, Hart and Rakoff (1944) have shown the effect for thiouracil, glutathione, cysteine, ascorbic acid, para-aminobenzoic acid, sodium sulfathiazole and sulfadiazine and Du Bois and Erway (1946) found that alpha-naphthyl thiourea is almost as effective as phenylthiourea in this respect while allyl-thiourea and thiourea are effective only in higher concentrations. The results of the present work are in agreement with this finding for, although 0.005 per cent phenylthiourea caused rapid and complete depigmentation of the embryo, thiourea, even at a concentration of 0.05 per cent, produced no discernible pigmentary changes.

It is of interest to note that in Figge's studies with the indophenol dyes these substances were found to produce, in addition to the pigmentary disturbances, specific effects on the eyes of some of the treated animals. The cells of the retina, lens and cornea were disoriented and the whole optic cup was collapsed and folded. No such effects have been observed in any of the animals subjected to the treatments employed in the present study. Even when the pigmented layer of the retina was completely lacking in melanin, the retinal layers themselves were normal in size and arrangement (Fig. 10). In this respect, therefore, the action of phenylthiourea differs from that of the indophenol dyes.

Summary

The leptodactylid toad, *Eleutherodactylus ricordii planirostris* (Cope), is unlike most anurans in that it possesses no aquatic larval stage. Its eggs are laid on land, beneath stones or logs. After about two weeks development within the egg, the young frogs hatch with the adult body form. It was found that eggs immersed in water will develop normally and at the usual rate providing the jelly layers and vitelline membranes are removed. In an attempt to ascertain to what degree the suppression of larval characters and the early assumption of the adult body form are dependent upon the activity of the thyroid gland, developing eggs were raised in solutions of thyroid-inhibiting drugs.

Embryos placed in 0.05 per cent thiourea or 0.005 per cent phenylthiourea on the fifth day of development failed to attain complete differentiation of the limbs and retained the larval tail, so that they were still embryonic in appearance 10 days after the tap-water controls had become complete little frogs. Animals raised in 0.005 per cent thiourea exhibited no retardation in limb development but did retain the larval tail so long as treatment was continued. Treatment with 0.001 per cent thiourea seemed to have no effect on development. Histological study of the thyroid glands of treated and control animals showed marked hyperplasia, hyperemia and reduction in colloid volume in the thyroids of specimens raised in 0.05 per cent thiourea or 0.005 per cent phenylthiourea. Thyroids of animals treated with 0.005 per cent thiourea showed slight hyperplasia and hyperemia but no significant differences in colloid volume as compared with controls. Thyroids of specimens raised in 0.001 per cent thiourea seemed to be unaffected. It appears that, in *Eleutherodactylus,* the loss of the larval tail and the complete differentiation of the limbs are features which are under thyroid control. On the other hand, the suppression or telescoping of many of the larval features cannot be attributed to thyroid activity since it occurs even under conditions of what seems to be extreme thyroid inhibition.

The embryos raised in 0.005 per cent phenylthiourea showed a rapid loss of pigment which involved not only the skin but also the pigmented coat of the eye. This is probably the result of the well-demonstrated inhibitory effect of this drug upon tyrosinase melanin formation.

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CAFFEINE EFFECTS ON FERTILIZATION AND DEVELOPMENT IN ARBACIA PUNCTULATA

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The effects of caffeine as a dietary factor upon the reproductivity and growth of mammalian species have been reported for rabbits by Stieve (1931), and for rats by Cheney (1944) and Bachmann et al. (1946), without uniformity in conclusions. None of these studies described the fundamental action of caffeine directly upon the gametes per se nor upon the early stages of development.

The effects of so many chemical factors in the environment upon the fertilization and development of the Arbacia egg are so well known, the comparative literature is so abundant, and the egg itself so ideal for studies of the effect of a substance upon permeabilities, cleavage rates, and developmental symmetry, that the Arbacia egg was chosen to investigate the critical factors underlying the influence of caffeine. The effects of caffeine were tested upon the gametes, zygote, and early developmental stages.

In 1945, it was reported by the author that caffeine, in concentrations of M/1000 (0.02 per cent) or above, depresses the O_2 consumption of the zygote as demonstrated by the Warburg-Barcroft micro-respirometer technique. It is the purpose of the current investigation to determine the relative sensitivity of the gametes to caffeine, its effect upon the cleavage stages and subsequent developmental phases through the mature pluteus larva, and the degree to which different concentrations retard and inhibit development.

Methods and Materials

Arbacia punctulata gametes, shed directly into sea water (SW) and into eight different concentrations of caffeine-in-sea-water (CSW), after fifteen minutes were mixed for fertilization and allowed to develop to the following selected stages: fertilization membrane (FM), streak stage, 2-, 4-, 8-celled stage, late cleavage, blastula, gastrula, and pluteus larva. All eggs per experiment were obtained from the same female and all sperm from one male. Observations were made over a 72-hour period for the comparison of the developmental rate and form in SW and CSW. Normal time rates for the development of Arbacia were accepted as stated by E. B. Harvey (1940). The molarity series was M/10, M/20, M/40, M/100, M/200, M/1000, M/5000, and M/10,000 corresponding in concentration equivalents to 2.0, 1.0, 0.50, 0.20, 0.10, 0.02, 0.004, and 0.002 per cent of the alkaloid (caffeine Merck U.S.P., $C_8H_{10}O_2N_4 \cdot H_2O$, mol. wt. 212.21). This same procedure was followed by the author (1946-a) in obtaining preliminary data.

The experimental series to determine the relative sensitivity of the several stages (FM through pluteus) to a caffeine environment, was conducted in the following manner. Fertilized eggs were allowed to develop under normal conditions in SW

to the desired stages. Then several hundred specimens of each of the first eight stages cited above were transferred to each of the eight concentrations of CSW. The most advanced stage of differentiation attained by the experimentals in each concentration of CSW, and the rate and total time required to reach this degree of development, were compared with control eggs from the same female developed normally in SW throughout the 72 hours. Equivalent fluid volumes in stender dishes were maintained at the temperature of running SW. Conditions of significance, such as volumes, temperature, pH, and pressure, were constant for controls and experimentals in each series. Observations were made microscopically under a water immersion lens at 440 ×.

TABLE I

Effect of Caffeine on Fertilization and Development of Arbacia ova

Gametes shed into medium SW or CSW for 15 min. before mixing. N = normal in time and form of development to pluteus. Key: SW = normal sea water; CSW = caffeine in SW; FM = fertilization membrane.

%	Molarity	I Control N ♀ X N ♂ Dev. SW	II Exper. N ♀ X C ♂ SW	III Exper. C ♀ X N ♂ SW	IV Exper. C ♀ X N ♂ CSW	V Exper. C ♀ X C ♂ SW	VI Exper. C ♀ X C ♂ CSW
0.002	M/10,000	N	N	N	N	N	N
0.004	M/5000	N	N	N	N	N	N
0.02	M/1000	N	N	N	Slight retard. in early cleavage.	N	Many abnormal gastrulae. No plutei in 72 hours.
0.10	M/200	N	N	N or slight retard. 4-cell on but pluteus normal form.	Retard. early cleavage. Arrested in late cleavage. No blastula. Cytolysis begun in 8 to 9 hours.	Normal blastula arrested in gast. Few early plutei —prism stage.	Ditto Col. IV with sharply localized echinochrome. Disintegration.
0.20	M/100	N	N	Time delay but plutei normal form.	Time retarded. Most div. abnormal. Arrested 4 and 8's when controls in 64 or late cleavage. Echinochrome localized. Dead.	Essential form N. Gastrula pluteus transition retarded several hours.	Ditto Col. IV with abnormal form in the 2-celled stage. Cells unequal.
0.50	M/40	N	Normal plutei time and form but pigment conc. noted and move slow.	Normal blast. Gastr. delay and arrest. Some cytolysis. No larva.	No FM or 1% FM. Egg oval. Pigment conc. begins in 2 to 3 hrs. and localized in 10 hrs. Then cytolysis.	Arrested in 16-cell to blast. Few abnormal gastrulae. No plutei before cytolysis.	Ditto Col. IV but develop pigment conc. in 5 to 7 hrs. Cytolysis.
1.0	M/20	N	Normal blast. but few reach gastr. No plutei.	Similar to Col. II but many abnormal cleavage and some cytol. in 6–7 hrs.	No FM. Eggs shape abnormal. Pigment clump in all within 1 hour. Cytolysis.	Early cleavage time retarded. Never beyond 16. Mostly less and abnormal.	Ditto Col. IV.
2.0	M/10	N	Time retard. 10% dev. into blast.	Time retarded. 16-cell maximum. Form abnormal. Arrested mostly in 4-celled. Pigment conc. Cytolysis.	No FM and ditto Col. IV for 1% given above.	Ditto Col. III but many arrested earlier in 2-cell. Maximum 8-celled rare.	Ditto Col. IV. No FM.

Results

Immersion of either or both the egg and sperm in any concentration of caffeine for 15 minutes prior to mixing the gametes did not render the egg non-fertilizable nor destroy the ability of the sperm to fertilize. These gametes, however, were not unaffected, at least by the higher concentrations, since with pretreated gametes (i.e. caffeinized eggs × caffeinized sperm), although they formed the FM when mixed and allowed to develop in SW, produced a zygote which never survived be-

Table II

Sensitivity to Caffeine Concentration

Dev. stage when transferred to caffeine	Term used is maximum development before death					
	0.02%	0.10%	0.2%	0.5%	1.0%	2.0%
I. Fertilization membrane	Gastrula to pluteus. Time retarded but form normal.	Blastula majority. Some gastrulae.	8 cells.	FM. No further dev.	FM. No dev.	FM. No dev.
II. Streak	Gastrula to pluteus. Time retarded but form normal.	Blastula majority. Some gastrulae.	16 cells.	No further dev.	No dev.	No dev.
III. 2-cells	Gastrula to pluteus. Time retarded but form normal.	32-celled and a few blastulae.	8 and 16.	No further dev.	No dev.	No dev.
IV. 4-cells	Gastrula to pluteus. Time retarded but form normal.	Blastulae fragmented badly.	16 and a few 32.	No further dev.	No dev.	No dev.
V. 8-cells	Gastrula to pluteus. Time retarded but form normal.	Blastulae less fragmented and a few gast.	16 and a few 32.	No further dev.	No dev.	No dev.
VI. Late cleavage	Gastrula to pluteus. Time retarded but form normal.	Blastulae.	Blast. move slow.	No further dev.	No dev.	No dev.
VII. Blastula	Gastrula to pluteus. Time retarded but form normal.	Gastrulae and "prism" triangles. No plutei in 3 days.	Gastr.	Abnorm. gastr.	Abnorm. gastr. Frag. badly.	No dev. Died in 3 hrs.
VIII. Gastrula	Gastrula to pluteus. Time retarded but form normal.	Triangular "prisms" only. No plutei, not even short prongs.	No dev.	No dev.	No dev.	No dev.

yond the early cleavage stages. Table I reveals the fact that typical plutei developed in normal time and form in all six combinations listed under columns I–VI inclusive with regard to molarities of M/5000 and M/10,000. The final line in Table I shows that the gametes, when shed into M/10 CSW (which approaches the maximum solubility concentration of this alkaloid in SW), and allowed to develop in SW, did form the FM but the zygote was retarded in its development and failed to form plutei before death. When shed into M/10 CSW and mixed for development also in CSW, the FM did not form, or if it did, it was not separated from the egg so as to be visible. Table I presents the various conditions under which the "first" retardation effects of any specific kind were observable. This table also shows the extent to which each concentration of caffeine interfered with the symmetry of normal growth.

The ability of the organism to carry on its normal metabolism in the presence of this trimethylated purine molecule is demonstrated by Table II, the Sensitivity Table. Eight typical stages of Arbacia development were transferred to a graded series of eight different concentrations of CSW. Table II lists only six molarities since it had been found in preliminary experiments by the author(1946-b) that M/10,000 and M/5000 CSW caused no response regardless of the organism's developmental stage when placed in CSW. All stages from FM through the Gastrula continued development to normal plutei in M/1000 CSW but the time schedule was retarded. The 2- and 4-celled stages were delayed more than the subsequent cleavage stages. M/200 CSW inhibited plutei formation from all stages. The maximum effect due to this concentration was similarly on the 2-celled stage and very few individuals ultimately exceeded the 32-celled stage. Other stages reached blastulation in M/200 CSW. Gastrulae formed "prisms" but no plutei. Higher molarities, M/100, M/40, M/20, and M/10 CSW, were more retarding and the last three (M/40, M/20, M/10 CSW) typically inhibited *all* further development beyond the stage attained when subjected to caffeine.

Discussion

Data by the author (1945) on the effect of caffeine upon the oxygen consumption of the fertilized Arbacia ova, recorded evidence that caffeine does affect the normal metabolism of the zygote. The effect of the molarities less than M/200 CSW in depressing the O_2 uptake of fertilized Arbacia eggs was slight and variable within 10 per cent, so they were not considered significant. Repetition of these experiments has emphasized again that molarities of M/200 CSW or above clearly depress the O_2 uptake as shown by Figure 1, the O_2 consumption—time relationship graph. These same molarities are the concentrations which are unquestionably effective in affecting the fertilization and the subsequent developmental time factor in the current study. In complete agreement are the effects of increasing concentrations of caffeine in depressing the O_2 uptake and in retarding the developmental time. The inhibition of the O_2 consumption effect of caffeine is undoubtedly a primary factor in the retardation of metabolism and cell division demonstrated by Table I. The possible mechanism by which caffeine interefers with the cellular metabolism, possibly by affecting only a single site in the pathway of the cytochrome oxidase-cytochrome chain of reactions, has been discussed previously by the author (1945).

There is some evidence that the egg is more sensitive than the sperm to caffeine. It will be noted by an examination of Table I that the lowest concentration, M/1000, at which any effect can be observed is under conditions where the caffeine-pretreated egg was fertilized by a non-treated sperm and developed in a CSW environment. The first effect with M/200 CSW involves the C♀ in M/200 CSW × N♂ and development in SW. The reverse, N♀ × C♂ M/200 CSW, with development in SW caused no retardation effect.

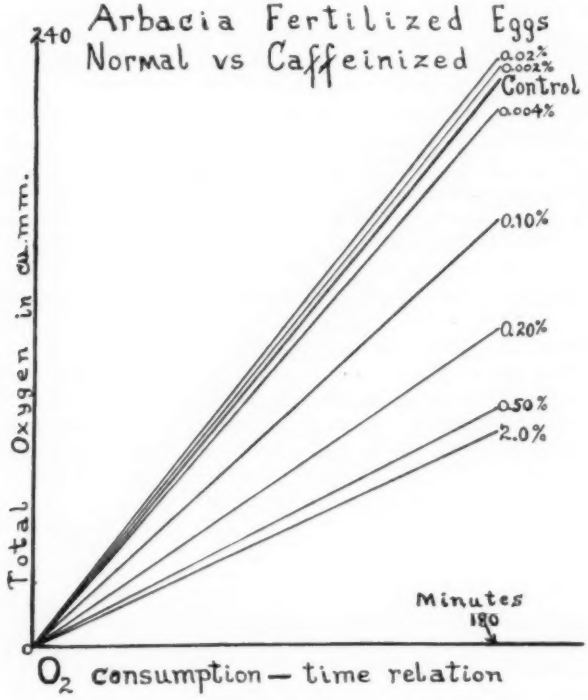

FIGURE 1

An osmotic effect of caffeine might be a cause underlying the sensitivity effects observed. Consequently, the eggs of a single female were subjected to the concentration series of the eight different molarities. Temperature was uniform by keeping the stender dishes in running sea water. The pH was not a factor since the differences between the normal SW, van't Hoff's artificial SW, and caffeine in either, did not increase the alkalinity more than 0.06 of a pH unit even in the highest concentration employed. This is well within the range, 6.0 – 8.3, within which the Arbacia egg is fertilizable and normal development occurs as shown by Smith and Clowes (1924). Since the unfertilized Arbacia egg is a sphere, egg diameters were measured directly by an ocular micrometer and volumes were calculated by the

formula, $r^3 \times 4/3\pi$. In the case of the unfertilized egg alone, a total of over 5000 ova in the eight experimental media were examined. Detailed results employing the most drastic concentration (2 per cent) are cited in Table III.

Obviously, no significant effect can be concluded from the facts presented in Table III for the unfertilized egg. Similar statistics indicated also negative results with reference to the fertilized egg. A slight osmotic effect with time, if due to caffeine, could conceivably alter the transport of water which could modify the internal metabolism of the egg.

TABLE III

Diameter and volume variation in caffeinized (2% = M/10) Arbacia eggs

Egg No.*	30 *Minutes* after shedding eggs into				2 *Hours* after shedding eggs into				7 *Hours* after shedding eggs into			
	Sea water		2% Caffeine-in-SW		Sea water		2% Caffeine-in-SW		Sea water		2% Caffeine-in-SW	
	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000
1	71.0	187	71.0	187	71.0	187	72.6	201	71.0	187	72.6	201
2	72.6	201	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201
3	71.0	187	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201
4	72.6	201	71.0	187	72.6	201	72.6	201	71.0	187	72.6	201
5	71.0	187	71.0	187	71.0	187	72.6	201	71.0	187	74.3	214
6	69.3	173	71.0	187	72.6	201	71.0	187	72.6	201	74.3	214
7	71.0	187	72.6	201	72.6	201	71.0	187	72.6	201	71.0	187
8	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187	72.6	201
9	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201	71.0	187
10	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187
Aver.	71.2	188	71.2	188	71.5	191	71.6	193	71.8	194	72.5	199
	30 Min. normal vs. caffeinized (2%) Diameter difference is zero Volume difference is zero				2 Hr. normal vs. caffeinized (2%) Diameter difference is 0.1 mu Volume difference is 1400 = 0.73% increase				7 Hr. normal vs. caffeinized (2%) Diameter difference is 0.7 mu Volume difference is 5300 = 2.75% increase			

No significant variation in 30 minutes, two hours, and seven hours.

* Each egg diameter listed is the average of 10 micrometer unit measurements on eggs from a single female. Final average of the 10 diameters cited in each column is actually the average of 100 eggs from the same female.

Advantage was taken of the procedures contributed to the literature by Harvey (1910), Lillie (1916, 1917, 1918), McCutcheon and Lucké (1926), Stewart (1931), and Jacobs and Stewart (1932), all of whom studied permeabilities employing the Arbacia egg as test material. Since rapid dehydration by strongly hypertonic solutions has destructive action on fertilized eggs, hypotonic solutions were used from which temporary immersion (30 minutes) results in complete recovery if transferred to normal SW.

Eggs from the same female were employed for both the unfertilized and fertilized series of experimental conditions. Several hundred ova, according to the series, were placed in stender dishes of normal SW (referred to here as NSW), van't Hoff's artificial sea water (ASW), bicarbonate—buffered to the pH of the NSW, hypotonic (40 per cent) sea water (40 ASW : 60 distilled water) indicated here as HSW, and HSW plus caffeine, i.e. CSW. The caffeine concentrations used were M/1000 (0.02 per cent), M/100 (0.20 per cent), and M/10 (2.0 percent). At the end of the first 30-minute period in any given experiment, approximately half of the eggs were transferred from CSW and divided approximately into equal masses to NSW and ASW for recovery studies. Readings (diameters) were made each minute for 10 minutes, followed by readings at 20, 30, and 60 minutes, and periodically for 24 hours although only the first 60 minutes were significant in the current study. Statistical tables similar to Table III were compiled. Molarities of M/1000, M/100, M/10 CSW in 40 per cent ASW were chosen because they represent significant concentrations as indicated by retardation effects of caffeine in Arbacia development—see Table I.

Since osmotic swelling is primarily one of diffusion, it is expected that when a cell is far from osmotic equilibrium with its environment, it will swell rapidly but the rate of swelling will decrease as equilibrium is approached. Volume changes were plotted against time. Thereby the velocity of swelling was noted. The rate of swelling was determined by the formula (McCutcheon and Lucké, 1926) in general use for this purpose; namely,

$$Kt = l_n \frac{V_{eq} - V_0}{V_{eq} - V_t}.$$

The experimental points were found to fall along a straight line as would be expected when

$$\log \frac{V_{eq} - V_0}{V_{eq} - V_t}$$

was plotted against time. The slope of the line gives the value of the velocity constant as k. This equation expresses the process of swelling in 40 per cent HSW and 40 per cent CSW for both unfertilized and fertilized eggs. This would be anticipated in a diffusion process.

The comparison of primary interest in the current paper is between data derived from the osmotic changes (rate and amount) specifically in HSW and the corresponding CSW. Comparison of these effects was also compared with the controls in NSW and ASW. No significant difference was evident in unfertilized eggs under the two hypotonic media of HSW and the corresponding CSW for any of the three concentrations of caffeine. Fertilized eggs show no difference under these experimental conditions. True, the fertilized eggs were more permeable than unfertilized eggs but the addition of caffeine had relatively no effect upon either series. *In other words, the HSW and the corresponding CSW results were equivalent.* Greater permeability to water and greater variability of fertilized eggs was expected since there is relative constancy of O_2 consumption before fertilization. The concentrations used have been shown earlier in this paper by the author to retard or inhibit cleavage completely. This fact indicates that the changes occurring in perme-

ability at the time of cleavage are of a different kind from those associated with cleavage furrow formation per se. The latter process is affected by caffeine which retards the O_2 uptake (Cheney, 1945).

The possibility of a viscosity effect of caffeine upon the Arbacia egg protoplasm, as a factor in explanation of the observed retardation of cleavage in caffeine solutions, has led to a study of the echinochrome stratification and egg deformation by centrifugation. The report upon these phenomena and the effect of caffeine upon the tension forces at the surface layers will be discussed in a later publication.

Summary

1. Caffeine does affect the gametes and the zygotes development of *Arbacia punctulata*.

2. Pre-treatment of both gametes with caffeine (0.5 per cent or higher) prior to mixing for fertilization and developed in CSW prevents the separation of the fertilization membrane.

3. Since some development occurs if both gametes are caffeinized but developed in SW, the FM may form but not be separated from the egg mass in the case of CSW as the environment.

4. The results of gamete activity following immersion and subsequent fertilization and development in CSW suggest that the egg is somewhat more sensitive than the sperm to caffeine.

5. Minimum effective concentrations in general are 0.1 per cent CSW (M/200). Slight retardation of development in 0.02 per cent CSW, under conditions of C♀ × N♂ and development in SW, is noted.

6. Oxygen uptake inhibition effect of caffeine is a primary factor in the retardation of cleavage time.

7. Early cleavage stages are retarded most.

8. The inhibition of O_2 utilization, retardation of cleavage time, and the degree of interference with the symmetrical differentiation attained, are directly proportional to the caffeine concentrations.

9. Concentrations of 0.5 per cent CSW (M/40) or higher as an environmental medium inhibit completely the physiological processes underlying the mechanics of further differentiation.

10. Caffeine does not initiate an osmotic effect with regard to the transport of water into the Arbacia egg.

11. Other factors in the explanation of the results observed are suggested.

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THE RESISTANCE OF METALLIC SILVER TO MARINE FOULING

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Copper has been commonly employed to resist the attachment of fouling growths on structures exposed to the sea, while the salts of both copper and mercury are commonly employed as toxic pigments in antifouling paints. The toxicity of solutions of silver salts to organisms such as barnacles has been shown by Clarke (1947) to be comparable to that of copper and mercury. Mr. F. L. LaQue of the International Nickel Company informs us that specimens of fine silver which he exposed at Kure Beach, North Carolina, for as long as 195 days remained practically free of fouling. Similar results were observed by Dr. W. F. Clapp with exposures at Marion, Massachusetts. On the other hand, the Kure Beach specimens when cleaned and re-exposed at Duxbury, Massachusetts, fouled as heavily as nickel or other inert metals tested at the same time. The reason for this difference in behavior is obscure. Mr. LaQue (in press) has classified silver among metals with variable or intermediate ability to resist fouling.

Because of the uncertainty existing concerning the fouling of silver, Mr. LaQue suggested that more systematic tests should be conducted and with the help of Mr. J. L. Christie of the Handy and Harman Company supplied the specimens used in the study to be reported. These consisted of fine silver containing no copper and of coin silver in which 10 per cent of copper is present as an alloy.

In the first experiment, summarized in Table I, two panels of pure silver, one panel of coin silver and one of copper were exposed simultaneously in the sea at Miami Beach, Florida, for one year. One of the silver panels had previously been exposed for two months to running sea water in the laboratory under conditions which permitted a black coating of corrosion products (presumably silver chloride) to form on the surface. The sterling silver and copper panels were 4×6 inches in dimensions, the coin silver 3×3 inches. The panels were held in a wooden frame which prevented metallic contact between them, and left almost the entire area of both sides exposed to the sea water.

Throughout the year's exposure the coin silver remained entirely free of fouling. It resisted attachment better than the sample of copper to which light fouling, consisting of barnacles and encrusting bryozoans, attached temporarily from time to time during the exposure. The copper, however, was entirely clean at the eleventh month, after which the specimen was lost. In contrast, the two specimens of pure silver commenced to foul within the first and second month and both were completely covered at the end of the fourth month of exposure.

One specimen of silver had been soaked in sea water prior to exposure in the sea in the expectation that fouling would be accelerated by the formation of a coating of corrosion products on its surface. This expectation was not realized. The

[1] Contribution No. 411.

TABLE I

Monthly fouling ratings on silver, copper and a silver alloy on marine exposure at Miami Beach, Florida, March 1, 1946–March 1, 1947

Months.....	1	2	3	4	5	6	7	8	9	10	11	12
Silver	3	2	2 1	4	4	4	4	4	4	4	4	4
Silver presoaked 2 months	0	1	2 0	4	4	4	4 3	4	4	4	4	—
Coin silver	0	0	0	0	0	0	0	0	0	0	0	0
Copper	0	0	0	1	1	1	1	0	0	1	0	—

Fouling Ratings

0 = no fouling
1 = <25% fouled
2 = 25–50% fouled
3 = 50–75% fouled
4 = 75–100% fouled

A double rating indicates a different degree of fouling for the two sides of a panel.

panel actually resisted fouling during the early months of exposure better than the specimen immersed with a clean surface of bright metal.

The resistance of copper to fouling was attributed by Sir Humphrey Davy (1824) to the solution of the metal. Subsequent studies (Ketchum, Ferry, Redfield and Burns, 1943) led to the belief that the ions formed on the solution of copper or its salts prevent the attachment of marine organisms by virtue of their poisonous action . Davy's conclusion was based primarily on the observation that copper did not foul when, through contact with a baser metal, a galvanic couple was formed which prevented the solution of the copper. In discussing the germicidal effects of silver, Goetz, Tracy and Goetz (1942) have pointed out that one must distinguish between the volume effect due to small silver ion concentrations in the water and the surface effect which occurs when microorganisms are brought into wetting contact with surfaces on which silver is absorbed. In anticipation of the possibility that metallic silver might resist the attachment of fouling organisms by virtue of some surface effect which would persist after solution was suppressed by galvanic action, a series of silver panels were exposed to the sea coupled with several metals standing higher in the galvanic series.

In this experiment, which is summarized in Table II, strips of copper, nickel, iron, and zinc, 1×6 inches in dimensions, were held in contact with 4×6 inch silver panels by means of the supporting rack. One uncoupled panel of pure silver was included as a control, as was a silver dollar, as a further check on the fouling of coin silver.

The results confirmed the other experiment in that the silver coin resisted fouling for the entire period of twelve months' exposure except for the temporary attachment of some early larval stages (incipient fouling) during the first two months.

TABLE II

Monthly fouling ratings of silver coupled with various metals on marine exposure at Miami Beach, Florida, January 1, 1946–January 1, 1947

Months....	1	2	3	4	5	6	7	8	9	10	11	12
Ag	1	1	1	1	4*	4*	4*	4*	4*	4*	4*	4*
Ag–Cu	1	1	0	0	4	4	4	4	4	4	4	4
Ag–Ni	2	3	4	4	4	4	4	4	4	4	4	4
Ag–Fe	4	4	4	4	4	4	4	4	4	4	4	4
Ag–Zn	4	4	4	4	4	4	4	4	4	4	4	4
Ag–dollar	½	½	0	0	0	0	0	0	0	0	0	0

* Principally a sponge covering entire panel and excluding other fouling from attaching.

Fouling Ratings

0 = no fouling
½ = incipient fouling
1 = <25% fouled
2 = 25–50% fouled
3 = 50–75% fouled
4 = 75–100% fouled

The pure silver, in contrast, was fouled lightly during the first four months after which it became generally covered with an encrusting sponge.[2]

The effect of coupling the silver to a baser metal was to shorten the time required for the silver to foul. When coupled with the more electro-negative metals, zinc and iron, the silver fouled heavily during the first month. Coupled to nickel the silver showed some slight resistance to fouling only during the first two months. Contact with copper had scarcely any effect on the fouling of the silver. The effects are thus proportional to the difference in potential of the coupled metals.

These results provide a useful control on the resistance of silver to fouling since they show that the light fouling of the uncoupled silver during the early months of exposure are not due to conditions unfavorable to fouling during this period. They warrant the conclusion that the temporary resistance of silver to fouling is dependent on the solution of the metal in the same sense as is the case with copper. There is no indication that the surface of silver, from which solution is prevented by coupling with zinc or iron, has any antifouling action. It should be pointed out, however, that these observations do not preclude in any way the possibility that surface effects such as discussed by Goetz, Tracy and Goetz (1942) might be influential in preventing attachment of fouling if suitable surfaces were established.

The most interesting and perhaps useful result of these experiments is the demonstration of the prolonged resistance to fouling of coin silver, which is apparently equal to copper and superior to pure silver. Coin silver contains about 10 per cent of copper, an amount much less than is present in other copper alloys which successfully resist fouling. The explanation appears to be in the high rate of solution of the coin silver when exposed in the sea.

The specimen of coin silver exposed at Miami lost weight during the year at an average rate of 16.8 mg/dm²/day, whereas the specimen of pure silver simultane-

[2] Mr. LaQue exposed duplicates of these specimens at Kure Beach, N. C., for 169 days during the summer of 1946. Neither the fine or coin silver showed any fouling. However, fouling at the test location was extremely light.

ously exposed corroded at an average rate of 5.6 mg/dm²/day. Thus, the rate of solution of the silver was three times as great in the case of the coin silver, which in itself may account for its more prolonged resistance to fouling. Moreover, since copper composed 10 per cent of the alloy, copper may be assumed to have been liberated at a rate of 1.68 mg/dm²/day. While this is a lower rate than is adequate to prevent fouling in the case of other copper alloys, it exceeds the rate of solution (1 mg/dm²/day) of copper from effective antifouling paints. It seems probable consequently that the resistance of coin silver to fouling results from the combined effects of the silver and copper ions which are liberated as it dissolves.

A duplicate specimen of coin silver, exposed to the sea at Woods Hole during the same period, lost weight at an average rate of 3.52 mg/dm²/day. This is less than a quarter of the rate of solution observed in the warmer waters at Miami. It seems quite possible that under conditions of exposure which result in such low rates of solution coin silver may foul more readily. Unfortunately, little fouling occurs under any circumstances at the point of exposure at Woods Hole so that this possibility is not tested. Variation in the resistance of specimens of silver to fouling may be expected to result from variations in the conditions of exposure, such as temperature, which influence the rate of corrosion, as well as to variations in the copper present in the alloy.

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THE LIPIDS IN COLPIDIUM CAMPYLUM

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Introduction

Various investigators have reported that *Colpidium* contains fat in the cytoplasm. Mast (1938) holds that it is neutral fat; Wilber (1942) maintains that it is chiefly fatty acid. Both investigators, however, base their conclusions on the staining of colpidia with Nile blue sulfate.

In the literature there is apparently no information based on microchemical analyses concerning the formation and types of lipids in *Colpidium*. An investigation was consequently undertaken to ascertain the types of lipids present and the manner of formation. This present report is the first in a series and it deals with the synthesis of lipids in *Colpidium campylum*.

Material and Methods

A sterile culture of colpidia was established (Glaser and Coria, 1930) and was cultivated in 3 per cent Difco proteose-peptone solution (Elliot, 1933). The proteose-peptone was rendered fat-free by extracting it with hot alcohol (Bloor, 1943, p. 40). Solutions of this fat-free medium were negative to all the tests for lipids. Colpidia were washed 3 times in fat-free proteose-peptone solution before transfer from ordinary to fat-free culture medium.

The population of the cultures was estimated by counting the number of colpidia in a small, weighed drop of medium and from that result calculating the number of organisms in 1 cc.

Five days after transfer (when the population reached about 44,000 colpidia per cc.) 1 cc. of the culture (fluid plus organisms) was transferred to 17 cc. of boiling alcohol-ether mixture and the whole was then made up to 25 cc. The alcohol-ether mixture extracted all the lipids (Bloor, 1943). Phospholipids were estimated colorimetrically (Youngburg and Youngburg, 1930), cholesterol colorimetrically (Bloor, 1916), and fatty acid by oxidation (Bloor, 1928). The procedure is somewhat as follows: For estimation of neutral fat and bound cholesterol the extracted material is saponified; this sets free the cholesterol and the fatty acids contained in fat and cholesterol esters. Tests for free cholesterol were made and were consistently negative. Since no cholesterol was found, the fatty acid values represent fatty acid combined in fat and/or free in the extract. It should be remembered that "the cholesterol esters are normally present only in traces. Hence, after phospholipids are separated, the fatty acids present may be safely referred to as fats." (See Bloor, 1943, pp. 42–43.)

A second sample from the same culture was carefully filtered 3 times through fat-free cotton waste and hard filter paper to remove all the colpidia. The filtrate was then analyzed for lipid material.

The differences between the values for the whole culture and for the filtrate were equal to the amounts of lipids in the colpidia themselves.

It is extremely rare that pure fats are found in microorganisms. Usually they are found as mixtures which are almost impossible to separate. "Analyses, therefore, are restricted usually to the fatty acids present and the determination of the ratio of saturated to unsaturated fatty acids" (Porter, 1946, p. 409). This method was followed in the present work, although the degree of saturation of the fatty acids was not measured.

Results

All tests for cholesterol were negative. Table I shows that there is consistently about .0002 mg. of lipid phosphorus per 1,000 colpidia. The table also shows that there is more lipid phosphorus in the culture fluid (2.5 mg./100 cc.) than in the colpidia (0.9 mg. in the colpidia from 100 cc.).

Table I

Table showing the amount of lipid phosphorus in the culture medium and in the colpidia

In order to convert lipid *P* to phospholipid (assuming it all to be lecithin) multiply the lipid *P* values by 25.

Culture	Colpidia per cc.	Lipid *P* in whole culture. Mg./100 cc.	Lipid *P* in fluid alone. Mg./100 cc.	Lipid *P* in colpidia from 100 cc. Mg.	Lipid *P* in 1,000 colpidia. Mg.
101	44,000	3.6	2.3	1.3	0.0003
102	47,000	3.3	2.3	1.0	0.0003
103	39,000	3.3	2.6	0.7	0.0002
104	47,000	3.6	2.8	0.8	0.0002
105	44,000	3.1	2.3	0.8	0.0002
Mean	44,000	3.4	2.5	0.9	0.0002

Table II

Table showing the amount of fatty acid in the culture medium and in the colpidia themselves

Culture	Colpidia per cc.	Lipid in culture. Mg./100 cc.	Lipid in fluid alone. Mg./100 cc.	Lipid in colpidia from 100 cc. Mg.	Lipid in 1,000 colpidia. Mg.
101	44,000	212	150	62	0.012
102	47,000	225	187	38	0.008
103	39,000	225	175	50	0.013
104	47,000	263	213	50	0.010
105	44,000	237	188	49	0.011
Mean	44,000	232	183	49	0.011

Table II shows that there is consistently about 0.011 mg. of fatty acid per 1,000 colpidia. This table also shows that there is much more fatty acid in the culture fluid (183 mg./100 cc.) than in the colpidia (49 mg. in the colpidia from 100 cc.).

Discussion

At first sight the value of 0.011 mg. of fatty acid in 1,000 colpidia seems large. Calculations were made to ascertain roughly the percentage of the wet weight which

is lipid. It was assumed that *Colpidium* is a prolate spheroid. The volume of the individual organism is then expressed: $V = 4/3\pi a^2 b$, in which a is the minor semi-axis and b the major semi-axis.

The mean length of 20 colpidia selected at random from the cultures was 0.064 mm., the mean thickness 0.036 mm. The minor and major semi-axes are then 0.018 mm. and 0.032 mm., respectively. Substituting these values in the equation above we get $V = 0.0000418$ mm^3 for a single colpidium. For 1,000 colpidia, $V = 0.0418$ mm^3.

Assuming the specific gravity of protoplasm to be 1.00, the weight of 1,000 colpidia is 0.0148 mg. The amount of lipid is then 24 per cent of the wet weight.

For the first time quantitative estimates of the kind and the amount of lipid in *Colpidium* are presented. The apparent absence of cholesterol is in accord with other investigations on the protozoa (Wilber, 1946 a and b). The evidence strongly suggests that colpidia do not synthesize cholesterol. Whether they assimilate cholesterol, if it is present in the culture medium, is not known. The high content of fatty acid is in accord with results obtained with other micro-organisms: the lipid in the diphtheria bacillus is mostly fatty acid (Chargaff, 1933); all the lipid in the typhoid bacillus is fatty acid (Akasi, 1939); in myobacterium fatty acids make up about 70 per cent of the fat (Pangborn, Chargaff, and Anderson, 1932).

The production of fat (sudanophil granules) from ingested starch and protein has been reported in various ciliates (Zingher, 1933). However, neither fat free nor sterile culture media were used in the investigations.

It is interesting to observe that the colpidia produce such large amounts of lipid that some is eliminated into the surrounding culture fluid. In the present experiments the ratio of *phospholipid in the whole culture/phospholipid in culture fluid alone* is 1.2 and that of *fatty acid in the whole culture/fatty acid in the culture fluid alone* is 1.3. The inverse ratios are 0.7 and 0.8 respectively. All the cultures used were approximately the same age and contained about the same density of population. It is, therefore, not possible at present to conclude whether these ratios are constant or whether they vary with age of culture and size of population. Such an investigation is now in progress.

In the higher organisms, it is known that fat is formed from protein (Atkinson, Rapport, and Lush, 1922; Eckstein, 1929). The present results indicate that there is a synthesis of lipid from protein in *Colpidium* also. The medium in which the organisms were grown contained no measurable lipids. The cultures were completely sterile, as indicated by regular bacteriological tests. After colpidia had grown in the fat-free medium there was appreciable lipid material in the organisms and in the culture fluid. The only source of food for the colpidia was protein (the proteose-peptone). It seems clear, therefore, that the growing colpidia broke down the supplied protein and reformed it into the lipids which were demonstrated by chemical analysis.

Summary

1. *Colpidium campylum* was established in sterile culture and grown in fat-free proteose-peptone solution.

2. The colpidia and the culture fluid in which they were grown were analyzed for fatty acid, cholesterol, and phospholipid.

3. No cholesterol was found. Large amounts of fatty acid were found in the organisms and in the ambient culture fluid. Phospholipid was found in the colpidia and in the culture fluid. There was more lipid in the culture fluid than in the organisms.

4. It is concluded that *Colpidium* synthesizes lipid from protein.

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SURFACE ILLUMINATION AND BARNACLE ATTACHMENT

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During the past twenty years the effect of light conditions upon the attachment of barnacles has received a considerable amount of attention. The investigations of Visscher (1928) have shown that barnacles attach more readily to dark-colored than to green, white, or other light-colored surfaces. This has since been confirmed by Edmondson and Ingram (1939). Pomerat and Reiner (1942) report greater numbers of attachments of *Balanus eburneus* to the underside of horizontal black plates than to opal plates during daylight. Fewer numbers attached to the upper surface of black plates than to the lower surface. The distinction between black and opal was not apparent during darkness. These authors also found that attachment to opal and transparent collectors were similar on both upper and lower surfaces. Confirmatory results were obtained by Pomerat and Gregg (1942) and by Edmondson (1944).

The observations of McDougall (1943) substantiate those previously mentioned. Nevertheless, although during the day time the rate of attachment was greater in shade than in sunlight, it was considerably lower at night upon any surface. This might suggest that the stimulus to attachment was not a uniform condition of relative shade but rather the contrast in lighting conditions present only during daylight. Nevertheless, attempts by Gregg (1945) to demonstrate a stimulus to attachment in the contrasting effect of adjacent light and dark surfaces were inconclusive.

Since 1942 investigators at Miami have repeatedly observed, in the case of *Balanus amphitrite niveus* Darwin and *B. improvisus* Darwin, not only a greater rate of attachment to darker surfaces during daylight, but also a diminished rate of attachment to all surfaces during the hours of darkness. Furthermore, attachments in darkness were found to be even less than attachments to light colored surfaces during daytime.

In the absence of the last mentioned observation, the lesser attachment to lighter colors in daylight and the equal attachments to all surfaces during darkness might be explained in terms of a negative phototropotaxis, using the terminology of Fraenkel and Gunn (1940). This would bring about movement away from light surfaces in daylight and would account for failure to distinguish any particular surface during night time. It would not, however, explain the greater number of attachments to *light* surfaces in daylight than in darkness. The theory of negative phototropotactic behavior is apparently supported by the laboratory observations of Runnstrom (1925), Visscher (1928b), Visscher and Luce (1928), Hertz (1933) and Neu (1933), who show that while the nauplius larvae swim towards the lighted side of a vessel, cyprid larvae swim away and attach on the opposite side, their activity being greater in green light than in other portions of the spectrum.

[1] Contribution No. 20 from the Marine Laboratory, University of Miami.

Observations communicated by Gregg (in literis) show that under conditions of weak artificial illumination at night, attachments to white plates are greater than to black, thus reversing the daytime condition. This and the previously mentioned anomalies, which are not readily explained by simple negative phototropotaxis, prompted the experiments here described, whereby the numerical distribution of cyprids between adjacent black and white surfaces of various sizes in daylight was investigated as a preliminary to further observations under carefully controlled artificial illumination.

Acknowledgments are due to Mr. James H. Gregg for his valuable assistance in setting up the experiments.

Methods

The apparatus consisted of a series of similar glass plates eight inches square, painted checkerboard fashion with alternating black and white squares of equal sizes. The size of the squares varied from the extreme case in which the plate was painted entirely black or entirely white, to the other extreme, where the plate was painted with 64 one-inch squares. The plates were suspended in a wooden frame just below the surface of the sea water at the Miami Beach Boat Slips in such a way as to lie in a vertical plane parallel to the direction of tidal currents.

The experiments were repeated at two different sites, one inside a covered dock and the other in the open at the side of a dock. In the latter case, one side of the rack holding the panels was facing the open sea and the other was facing the darker underside of the dock. The arrangement of plates with respect to each other was a random one determined by removing numbered cards from a hat. By painting the reverse side of each plate with a different system of squares a different sequence of plates was provided on the two sides of the rack, thus providing two sets of observations.

The rack of plates was suspended in the covered dock from 9 A.M. to 5 P.M. on a calm, sunny day. On the following day, under the same weather conditions, it was exposed for a similar period in the open dock so that a total of four sets of observations was taken. Counts of the attached cyprids were made immediately upon removing from the water.

Results

Individual observations are recorded in Table I and the average of the four exposures is recorded at the end of the table. In the average of four exposures, the average density of attachments upon the entire surface of each panel, including both black and white areas, varied from the panel of one-inch squares with 5.7 per square inch to the panel of four-inch squares with 4.7. The attachments on an equal area of the panel consisting of an eight-inch black square were 4.6, Figure 1. The checkered panels as a whole, therefore, acquired attachments somewhat greater in density than the single black panel, with a decrease in average density as the size of squares increased. On the plain white panel the average attachment density was 1.4 per square inch, or considerably less than in the case of the checkered or black panels.

Table I shows that with decreasing numbers and increasing size of squares the numbers of attachments to white surfaces decreased. Thus, on the plate with one

TABLE I

Variation in density of barnacle attachments upon black and white squares of checkerboard collectors in relation to size of squares
Expressed in attachments per square inch

Experiment	Surface	Size of squares			
		8 inch entirely black or white	4 inch	2 inch	1 inch
Covered dock Side A 2/16/44	Black	4.4	5.8	4.1	4.6
	White	0.7	2.2	4.4	4.6
	Average entire panel	2.5	4.0	4.2	4.6
	Ratio black/white	6.6	2.6	0.9	1.0
Covered dock Side B 2/16/44	Black	3.6	6.1	6.0	5.5
	White	1.2	2.5	4.8	4.4
	Entire panel	2.4	4.3	5.4	4.9
	Ratio black/white	3.3	2.5	1.3	1.2
Open dock Side A 2/17/44	Black	2.9	5.2	3.5	4.6
	White	1.1	2.2	4.4	6.0
	Average entire panel	2.0	3.7	3.9	5.3
	Ratio black/white	2.6	2.5	0.9	0.8
Open dock Side B 2/17/44	Black	7.3	8.8	6.4	6.8
	White	2.6	5.0	6.0	9.3
	Average entire panel	4.9	6.9	6.2	8.0
	Ratio black/white	2.6	1.7	1.6	0.8
Average of 4 exposures	Black	4.6	6.4	5.0	5.4
	White	1.4	3.0	4.9	6.1
	Average entire panel	3.0	4.7	4.9	5.7
	Ratio black/white	3.3	2.2	1.0	0.9

inch squares, the average number of attachments to white areas was 6.1 per square inch. The density of attachment to an equivalent area on the plain white plate, consisting of one eight-inch square only, was 1.4. This is less than one quarter of the attachment to one-inch squares. On the plates with two- and four-inch squares the attachments were intermediate in number.

Attachments to black squares showed less variation. The attachment was greatest on the 4-square panel with four-inch squares, where the average density was 6.5, and least on the plain black eight-inch panel, with an average of 4.6 per square inch, or about three-quarters of the attachment to four-inch squares. Thus not only was variation considerably less than in the case of white squares, but it did not bear a continuous relation to the size of squares, and may not be significant.

It follows from the variation in attachments to white squares that the ratio of attachments upon black areas to attachments upon white areas decreased with diminishing size of squares. It was greater than 3 on the eight-inch squares whereas on the one- and two-inch squares black and white attachments were almost equal.

In order to demonstrate the absence of simple tropotactic response to surface illumination, the ratio of attachments to black over attachments to white is plotted

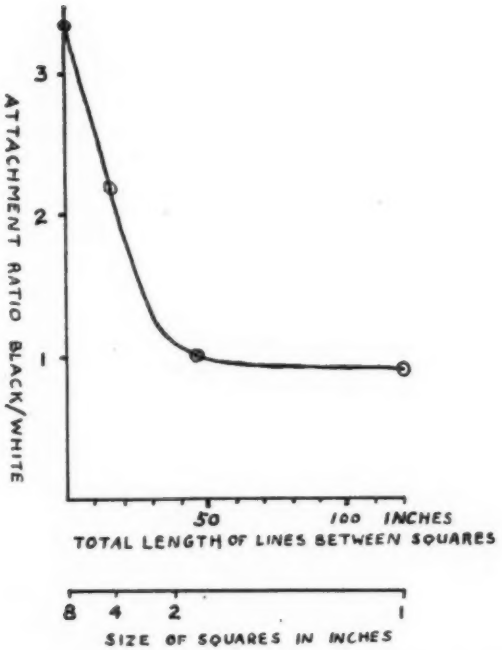

FIGURE 1. Ratio of attachments to black and to white squares in relation to size of squares and to length of black-white boundary lines.

in Figure 1 against the linear extent of the lines separating black and white squares on each plate. From this figure it is apparent that the ratio diminishes as the black/white separation length increases, reaching unity at a point corresponding to a square of side about 2 inches.

An examination of the data for the individual experiments shows a similar behavior in each case. The panels exposed in the covered dock did not differ significantly in numerical attachments, but showed a slightly lower attachment to large white squares, and consequently a slightly higher black/white ratio for the larger squares. It is doubtful whether the difference is sufficient to be of significance.

DISCUSSION

It is apparent that the results do not uphold the simple conception that a cyprid swims actively towards dark surfaces only or swims away from white surfaces, or is capable of orientation so as to move towards a black surface in preference to a white surface. The results indicate rather that with increasing opportunity of exerting such a "choice" the cyprids show an increasing lack of ability to discriminate between black and white surfaces (Fig. 1). The apparent discrimination is limited to squares over 2 inches in size.

The attachments to black surfaces appear to be little affected by adjacent white surfaces except possibly in the case of four-inch squares. A greater density of attachments to black is associated with the presence of four-inch white squares rather than with black alone, or with black coupled to white squares smaller than four inches. The difference is rather small, however, and may not be significant.

The most striking result of the experiments is the very considerable decrease in attachment density on white squares as the size of squares increases. This is in harmony with the possibility that the cyprid is influenced not by directional light rays from the panel surface but by the general light flux or intensity of diffuse light at some distance from it. In this manner diffused light resulting from scattered reflections adjacent to larger white squares would be more intense and more effective than diffused light produced by reflection adjacent to the smaller white squares where the black surfaces would be sufficiently close to exert a "diluting" effect on the light flux. On the basis of this explanation the cyprids may be stimulated to attach when they enter into an area characterized by low intensity of diffuse light, or they may be inhibited from attaching to an increasing extent as the intensity of diffuse light increases. They would thus be subjected to the inhibiting effect as they approached large white squares, but smaller white squares as indicated above would be less effective because of the increasingly fine admixture of black surfaces which would provide a light flux in the immediate neighborhood approximating to that which a hypothetical gray surface would produce.

Greater attachment density to the small size checkerboards than to plain black surfaces may possibly indicate that there is an optimum intensity of diffuse light represented by that of the small square checkerboards equivalent to that produced by gray surfaces and that unrelieved black panels are less effective in providing attachments because the light intensity is below the optimum. The existence of an optimum light intensity would also explain the low attachment density characteristic of panels exposed during darkness which would be below the optimum. The anomalies reported by Gregg whereby, under conditions of weak artificial illumination at night, barnacles attach in greater numbers to white than to black panels may also be explained by the existence of an optimum intensity of light for attachment. White squares under weak artificial illumination might provide diffuse light of intensity nearer the optimum than black, whereas in daylight white would produce light above the optimum, black slightly below the optimum, and small checkerboards or gray would produce approximately the optimum. It is unfortunate that conditions did not permit an accurate determination of the illumination. It may be possible to remedy this in future experiments in which it is proposed to provide controlled artificial illumination during hours of darkness.

The experiments of Visscher and others (op. cit.) have demonstrated a simple negative phototropotaxis under laboratory conditions which is not borne out by the present investigations. The apparent contradiction, however, may be readily resolved when the natural conditions of submarine illumination are taken into consideration. As Whitney (1941) and Schallek (1942) have pointed out, light is usually polydirectional under sea water and only rarely is there a truly unidirectional beam. The sediment present under coastal and estuarine conditions where barnacles naturally occur serves to intensify the diffuse nature of the light. While working in diving helmets in 15 feet of water at the site of the present experiments it was found most difficult to orient with respect to the shaded and open part of the

dock. The intensity of illumination appeared to be almost equal in every direction, yet after taking a few steps into the shade of the dock the intensity would suddenly decrease very considerably. Even at a point immediately below the edge of the dock little difference was experienced in the light intensity when facing towards or away from the covered area.

It is possible that other and more reasonable interpretations of the experiments presented here may exist, but it is believed that the results, at least, indicate the necessity of a renewed examination of the light reactions of barnacle cyprids during attachment, preferably by means of carefully controlled laboratory experiments in which both diffuse and unidirectional light are considered and in which the possibility of a low optimum intensity favoring attachment is borne in mind.

Summary

1. Experiments were designed for the purpose of investigating the various anomalies reported in the response of barnacle larvae to black and to white surfaces. The apparatus consisted of panels of similar size but checkered with black and white squares of sides varying from eight inches to one inch.

2. Average attachment density over the entire panel increased slightly with diminishing size of squares. Attachment density to white squares increased with diminishing size of squares. Attachments to black squares did not vary continuously with size of squares. The distribution ratio between black and white decreased from over 3 on eight-inch squares to unity on 2-inch squares.

3. As an explanation of the results and of previously reported anomalies it is suggested that cyprids are stimulated to attachment by a low optimum light intensity of light, and that diffuse light rather than unidirectional light is the principal factor involved.

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COMPARISON OF THE BINDING ABILITY OF HEMOCYANIN AND SERUM ALBUMIN FOR ORGANIC IONS

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Introduction

The presence of hemocyanin in a dispersed form in the plasma of invertebrates suggests that this protein may act not only as a respiratory pigment but also as a transport vehicle for small ions. In mammalian plasma these functions are divided between two proteins, hemoglobin and serum albumin, respectively. The importance of the "vehicle function" of plasma albumin in facilitating or hindering the distribution of substances amongst various tissues and organs has been pointed out by Bennhold (1938) and by Davis (1946). No investigations have been reported on the interactions of hemocyanins with small ions, other than those involved in the respiratory function (Redfield, 1934; Dawson and Mallette, 1945).

A series of studies has been made, therefore, on the binding of several organic dye ions by the serum of the horseshoe crab, *Limulus polyphemus*. Parallel investigations for some anionic substances and bovine serum albumin have been reported previously (Klotz, 1946; Klotz and Walker, 1947). In the absence of quantitative data on the binding of cationic dyes by albumin, some experiments were carried out also with these substances and bovine serum albumin.

Methods and Materials

Quantitative measurements of the extent of binding by the proteins were made by the dialysis-equilibrium technique (Klotz, Walker and Pivan, 1946). A cellophane bag containing a measured quantity (10 cc.) of the serum, or of the solution of pure protein, was immersed in a large test tube containing a definite volume (20 cc.) of the solution of the small ion at a known concentration. The bag, impermeable to protein, was mounted on a glass frame from which a small bead was suspended by a Fiberglas thread into the protein solution. The test-tube was placed in a shaking device for twenty-four hours for the attainment of equilibrium. The bead in the protein solution served as a stirring device for the contents of the bag and thereby hastened materially the attainment of equilibrium. The bag was then removed and the external solution analyzed colorimetrically for the ion.

For each ion concentration, a control tube was prepared also, which differed from the primary tube only in that the former contained buffer rather than a protein solution or serum inside the bag. By this method it was possible to minimize any errors arising from binding of the ion by the cellophane membrane.

[1] The grant of a fellowship by the Lalor Foundation for research at the Marine Biological Laboratory during the summer of 1947 is gratefully acknowledged.

[2] This investigation was supported in part by a grant from the Graduate School Research Fund of Northwestern University.

For each ion investigated, a preliminary series of experiments was run to ascertain the time necessary for the attainment of equilibrium. With the bead device described, a period of twenty-four hours was found to be more than sufficient in every case. A typical set of results for the cationic dye, chrysoidine, is illustrated by the data in Table I. The other substances showed similar behavior.

TABLE I

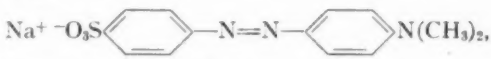
Time for attainment of equilibrium in binding of chrysoidine

Time	Conc. of dye outside bag
0.00 hours	9.2×10^{-5} moles/liter
6.25	5.2
18.75	5.2
23.5	5.2

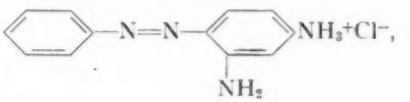
Limulus serum was obtained from a heart puncture of the animal.[3] Coagulation was permitted to occur at room temperature for about fifteen minutes. The serum obtained by subsequent filtration was diluted to four times its initial volume and stored in a refrigerator until used.

Bovine serum albumin was recrystallized material obtained from Armour and Company. Solutions of 0.2 and 2 per cent concentration were used in the binding experiments.

The methyl orange,



was a commercial sample of reagent grade. The chrysoidine,



was a commercial sample.

The dialysis experiments at pH 5.0 were carried out in 0.1 M acetate buffer solutions and those at pH 5.2 in 0.2 M phosphate. The pH values were measured with a Coleman glass electrode. Colorimetric analyses were carried out with a Klett-Summerson photoelectric colorimeter. All equilibria were attained in a water bath at $25 \pm 2°$ C.

RESULTS AND DISCUSSION

Data on the binding of methyl orange, a typical aromatic organic anion in its protein-binding properties (Klotz, 1946), by *Limulus* serum at a pH of 5.0 are summarized in Table II. It is evident that appreciable binding occurs with *Limulus* serum also, and that the extent of combination increases with the concentration of free dye. Relatively wide variations in the data are observed with this serum, however, probably because of differences between samples.

If one assumes that the serum contains about 4 per cent hemocyanin, then 10 cc. of the diluted solution within the bag contains approximately 0.1 gram of protein,

[3] We are indebted to Miss Marguerite Webb for technical advice.

TABLE II

Binding of methyl orange by Limulus *serum, pH 5.0, acetate buffer*

Conc. of free dye	Moles dye bound by hemocyanin in 10 cc. of diluted serum
1.5×10^{-5} M	0.5×10^{-7}
2.7	1.6
3.8	2.1
3.8	2.1
5.3	3.3
5.6	2.7
5.8	1.8
10.0	6.9
10.8	3.9
10.8	6.3

and at a concentration of 1×10^{-4} molar free dye, approximately 0.5×10^{-5} mole of methyl orange would be bound by one gram of hemocyanin. This is about one-tenth the degree of binding observed with bovine albumin (Klotz, Walker and Pivan, 1946) where approximately 6×10^{-5} mole of methyl orange is bound by one gram of protein at a free dye concentration of 1×10^{-4} molar.

Several experiments were carried out with methyl orange and hemocyanin in a phosphate buffer at pH 5.2. The results obtained (Table III) indicate a slightly decreased degree of binding. However, the difference in the two pH's is too small to be attributed to changes in hydrogen-ion concentration for there may be small deviations due to differences in the ionic nature and strength of the buffer solutions.

TABLE III

Binding of methyl orange by Limulus *serum, pH 5.2, phosphate buffer*

Conc. of free dye	Moles bound dye
4.8×10^{-5} M	1.2×10^{-7}
6.1	1.6
8.6	2.4
11.9	3.6
17.6	9.3

In the case of albumin, there is strong evidence (Klotz and Walker, 1947) that electrostatic attraction between the organic anion and a positively-charged, quaternary nitrogen of one of the basic amino acids contributes a major portion of the binding energy. While the demonstration of a similar force in the hemocyanin complex requires further work on the effect of pH, it is, nevertheless, of interest to note that this protein like albumin is rich in the basic amino acids, histidine, lysine and arginine (Dawson and Mallette, 1945).

As an example of a cationic species, the dye chrysoidine was used at a pH of 5. No significant binding was observed, as is evident from the data in Table IV. The concentration of free cation was not reduced significantly by the presence of diluted *Limulus* serum inside the cellophane bag.

In the absence of comparable data for albumin, a series of binding experiments was carried out with chrysoidine and bovine serum albumin. The results obtained have been assembled in Table V. It is evident that chrysoidine is bound by albumin,

though only about one-tenth as strongly as is methyl orange. If the same decrease in binding in going from the anion, methyl orange, to the cation, chrysoidine, holds in hemocyanin as is found with albumin, it would require more precise techniques than were available to detect the presence of a chrysoidine complex of hemocyanin.

TABLE IV

Absence of binding of chrysoidine by Limulus *serum, pH 5.0*

Conc. of free dye in equilibrium with protein	Conc. of free dye in blank
5.8×10^{-5} M	6.1×10^{-5} M
17.8	16.3
27.0	28.8
32.8	33.0
39.0	39.5
44.5	44.0

TABLE V

Binding of chrysoidine by bovine serum albumin, pH 4.7

Conc. of free dye	Moles bound dye / Moles total albumin
1.1×10^{-5} M	0.0523
2.4	0.136
3.8	0.188
5.8	0.279
6.0	0.294
12.0	0.577
12.7	0.652
19.8	0.871
19.8	1.09
24.5	1.05
29.5	1.31
34.5	1.42

The binding data for chrysoidine with bovine albumin can also be expressed analytically by the equation

$$\frac{\text{moles protein}}{\text{moles bound dye}} = \frac{1}{k\,n}\,\frac{1}{(\text{free dye})} + \frac{1}{n}$$

where k represents the intrinsic binding constant and n, the maximum number of chrysoidine ions which can be bound by a single albumin molecule. As has been shown previously (Klotz and Walker, 1947) this equation is derivable from the law of mass action for the ideal situation in multiple binding, i.e. one in which a bound ion exerts no electrostatic influence on successively bound ions. Thus within the precision of the present experiments, chrysoidine fulfills this ideal condition. The maximum number of sites, n, available to chrysoidine turns out to be 16. The intrinsic binding constant equals 3.1×10^2. From these values it follows (Klotz, Walker and Pivan, 1946) that the energy of binding of the first chrysoidine ion is about 5,000 calories/mole in comparison to 5,960 calories/mole for methyl orange.

Attempts were made also to obtain some information on the binding of methylene blue by albumin and hemocyanin, respectively. Unfortunately, the persistence

of micelle formation by this dye even at exceedingly low concentrations made it difficult to interpret the data. Difficulties were also encountered in preliminary experiments on the binding of ferric iron, apparently because of oxidation of the cellophane membranes in the presence of this ion at the relatively high acidities (pH 3) which were necessary to avoid formation of colloidal iron oxide.

Conclusions

The results obtained indicate that hemocyanin is capable of forming complexes with organic anions though the binding affinity is less than that observed with bovine albumin. Thus in addition to its function as a respiratory pigment, hemocyanin may be capable of acting as a vehicle in the transport of anions in the blood of invertebrates, as an agent for conserving desirable substances as the blood passes through the excretory organs and as a buffer against the effects of cytotoxic agents (Davis, 1946). In all of these actions, however, the present experiments indicate that its effectiveness is much less than that of serum albumin.

Summary

1. Hemocyanin forms complexes with organic anions, such as methyl orange, though with an affinity of about one-tenth that observed with the same dye and serum albumin.

2. No binding was observed between hemocyanin and the cationic dye, chrysoidine. Under comparable conditions bovine serum albumin combined with chrysoidine, thought quantitative calculations indicate an energy of binding about 1,000 calories less than that observed for albumin-methyl orange complexes.

3. The data indicate that hemocyanin acts not only as a respiratory pigment in the blood of invertebrates but also may serve to a limited extent in the distribution and conservation of organic ions among various organs and tissues.

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DEVELOPMENTAL CHANGES IN THE VIABILITY OF SQUID EMBRYOS AFTER SUBJECTION TO CYANIDE

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Introduction

As a consequence of data which have been accumulating for the past century, it is now well recognized that various animals typically considered as obligate aerobes are capable to a greater or less extent of withstanding exposure to oxygen lack (cf. the reviews by Slater, 1928, and v. Brand, 1946). This ability is not necessarily a static characteristic throughout the lifetime of an organism, however, for it has frequently been reported for example that the new born mammals of various species are definitely more resistant to asphyxia than are adults.

Much of the literature in recent times on the changing susceptibility of organisms to oxygen lack has dealt with the effects of anoxia upon such early embryonic processes as segmentation, gastrulation and neurulation (for bibliography see Needham, 1931). There are also, however, data which leave no doubt that various organisms exhibit changes in susceptibility to anoxia throughout much later phases of development. Amerling (1908) has noted that as the body length of Rana and Bufo tadpoles increases to 12 mm. there is a gradual decline in the resistance to oxygen lack. Similarly Burrows (1921) observed that whereas heart-fibroblasts from 4–5 day chick embryos could grow and contract in pure nitrogen for as long as 24 hours, those of 10–15 day embryos would exhibit no activity whatever under similar circumstances. More recently, the original observations on mammals have been confirmed and extended in an attempt to learn something of the chemical mechanism involved (Reiss, 1931; Selle, 1944; Himwich, Bernstein, Herrlich, Chesler and Fazekas, 1942; Glass, Snyder and Webster, 1944). It is now clear too that similar changes in ability to withstand asphyxia occur throughout the devolpment of the duck (Rostorfer and Rigdon, 1947).

Most of these researches dealing with anoxia, however, have been concerned either with very early or very late stages of development, or with adult animals. It was felt that it might be of some value to examine resistance to oxygen lack in a more systematic fashion during the period of development, which follows the establishment of the primary organ-systems. To do this, since the attainment of strict experimental anaerobiosis is a somewhat difficult matter, it was deemed desirable to render oxygen unavailable to the experimental animals by the use of a suitable respiratory inhibitor. Cyanide is such a compound for it is generally conceded to produce its primary inhibitory effects upon animal tissues by a depression of oxidative metabolism. Inasmuch as it interferes with the intracellular transport of oxygen, it imposes essentially the same conditions upon the organism as does oxygen lack.

A series of studies has, therefore, been undertaken to examine the resistance of several species of Metazoa to the action of cyanide at various stages throughout de-

velopment. The present paper deals with the effect of N/1000 CN upon the general viability of the squid, *Loligo pealei*, from about the fourth day of development to maturity. This concentration of cyanide causes in most tissues a maximal inhibition of oxygen consumption (Dixon and Elliot, 1929). To provide correlative data, a few measurements were made at various stages of the normal oxygen consumption and the effect of cyanide on it, and of the normal carbon dioxide production. In a few instances organisms were subjected to relatively high concentrations of carbon dioxide in order to determine whether or not the respiratory pigment of the blood was necessary for life at the developmental stage in question.

It was considered that experiments of this kind on the squid would be of particular interest in view of the very pronounced susceptibility of the adult of this species to anoxia.

Material and Methods

Mature squid (*L. pealei*) of both sexes were obtained fresh from the nets or live-boxes of the Collecting Department of the Marine Biological Laboratory and were placed in a large aquarium of running seawater containing anchored algae. The marine minnow, *Fundulus heteroclitus*, was used as food and the tank was covered and kept dark until eggs were deposited. In this way the animals remained healthy, eggs in abundance were obtained in one to two days, and the time at which the eggs were deposited could be determined to within a few hours.

The eggs from each female were kept separately in gently running seawater. After the first day or two of development the strings were separated from one another, freed of the two outermost gelatinous envelopes, and cut into ½–1-inch lengths to permit the same rate of development in all embryos.

Samples of normal embryos were fixed in saline formalin (Microtomist's Vade-Mecum, p. 458) at the time of each experiment. Their stage of development was determined by comparison with the figures of Naef (1928) for *Loligo vulgaris* rather than with those published earlier for *L. pealei* because Naef's monograph is more commonly available. This procedure seems permissible since, according to Naef, there is little or no evidence that the species are distinct.

The time scale of development which will be employed in presenting the observations allows only two-thirds of a day per stage instead of one day as in *L. vulgaris* (Naef, 1928). This presumes a total of 12 days from oviposition to hatching which is the usual period cited at Woods Hole for the American squid (Grave, unpublished). The actual numbers of days required for five typical batches of eggs to hatch along with the corresponding temperatures are given in Table I.

Table I

Laid	Hatched	Days required for development	Extremes of temperature for the period of development
June 8	June 30	22	14.2–17.2
June 12	July 5	23	15.6–18.6
June 13	June 26	13	15.8–16.9
July 19	Aug. 3–5	15–17	17.8–21.1
July 21	Aug. 4	14	17.8–21.1

The concentration of the Stock solution of cyanide was standardized at intervals, and prior to an experiment the pH of the N/1000 solution of cyanide in seawater to which the organisms were to be exposed was, if necessary, adjusted to the pH of seawater.

The respiration experiments were performed by the direct method of Warburg (Dixon, 1943). Twenty or thirty embryos were placed in each respirometer vessel. The approximate volume of the animals was previously determined by placing them in a small graduated cylinder and adding a measured quantity of seawater.

In determining the effect of cyanide on the rate of oxygen consumption, each experiment usually included two controls and two or more vessels to which was added sufficient N/100 cyanide at the pH of seawater to bring the cyanide concentration to approximately N/1000. In some experiments the inhibitor was added at the beginning. In others the vessels were removed from the manometers after a preliminary determination of the normal rate of oxygen consumption and the inhibitor then added. With cyanide present the insets contained the mixture of sodium cyanide and sodium hydroxide suggested by Krebs, 1935.[1] The temperature in the initial experiments varied from one experiment to another over the range 21–25° C. Later it was maintained constant at 22° C. The duration of the respiration experiments varied from 3½ to 8½ hours.

The rate of oxygen consumption and carbon dioxide production are expressed on the basis of number of embryos rather than weight of respiring tissue because of the presence at most stages of development of the jelly and of a large amount of inert yolk.

Solutions of carbon dioxide were made up immediately before use by adding various quantities of seawater previously saturated with carbon dioxide to about 8 liters of fully aerated seawater. The animals were quickly introduced, a protecting screen was submerged over them and the surface of the water was then covered with a thick layer of paraffin oil. Where both embryos and adults were used together, the former were confined in a piece of glass tubing of approximately 1½ in. bore, the ends of which were closed by a single layer of cheese cloth.

The authors are indebted to Virginia S. Black for determinations of the carbon dioxide partial pressures by the method of Krogh (1908).

Results

A. Viability after exposure to cyanide

When squid embryos are placed in dilute solutions of cyanide, certain characteristic symptoms very soon make their appearance. If the embryo normally shows rotation within the jelly and pulsation of the yolk sac (which is first seen in Stage XIV), these activities become slower and after approximately 10 minutes, cease. The hearts however continue to beat in cyanide though at a gradually declining rate for as long as two hours. The "oxidative reserve" available to the heart is clearly of greater magnitude than that maintaining the pulsation of the yolk sac.

[1] According to the data published by Robbie (*Jour. Cell. and Comp. Physiol.*, vol. 27, pp. 181, 1946), the cyanide concentration surrounding the organisms in the present experiments probably increased with time due to the uptake of carbon dioxide by the cyanide-hydroxide mixture in the inset. Since no appreciable change of inhibition occurred with time even the initial concentration of .001 N must have produced a maximal effect.

In embryos near hatching, and in larvae or adult squid, immersion in cyanide almost immediately produces acute respiratory distress, as indicated by greatly increased amplitude of mantle and siphon movements. Soon afterwards these movements cease. In addition, the chromatophoral system undergoes a characteristic cycle whereby the animals flush at first to a deep red or purple, then blanch permanently to a bluish-white color.

The criteria used for recovery were, in general, the reverse of the symptoms outlined above:

(a) In young embryos rotation within the egg capsule, pulsation of the yolk sac, and continuation of external development.

(b) In young hatching larvae and adults, resumption of normal body color (as opposed to the blanched condition), recovery of respiratory movements, and the ability to swim freely.

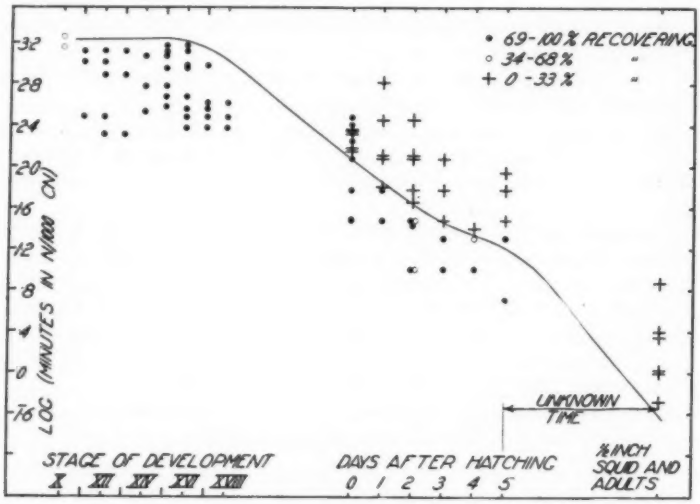

FIGURE 1. The number of organisms which recovered after subjection to cyanide for the periods indicated. Each entry represents a single experiment in which, as a rule, at least ten organisms were used.

It should be mentioned here that for the purposes of this paper, observations on "recovery" do not deal with the effect of cyanide upon subsequent morphology. Subjection to the inhibitor for long periods of time (i.e., 10 hours) frequently appears to have a subsequent teratological effect upon squid embryos which becomes evident at the time of formation of mantle and siphon. There may also be distortion of the yolk sac, though the embryo apparently continues to derive nourishment from it.

In a general way recovery is most rapid in the embryo, less so in the larvae, and least rapid in the adult. Squid younger than two days post-hatching show remark-

able "reversibility" when cyanide is replaced by normal seawater. In one case, mantle pulsations began within 8 minutes, and at this time the rate of the heart was very appreciably accelerated. One group of embryos which had been in cyanide for 17 hours recovered after two hours in seawater. Another group, subjected to cyanide for over 11 hours, resumed rotation and yolk-sac movements after only 5 minutes in seawater.

In Figure 1 are shown the viabilities of squid of various stages after immersion in cyanide for varying lengths of time. Each entry on this chart represents a single experiment in which, as a rule, at least ten organisms were used. If no more than one-third of the organisms subjected to cyanide survived, the entry for this experiment is shown as a cross; if between one- and two-thirds survived, the entry is a circle; and if between two-thirds and all of the organisms were viable, the entry is made as a point. Over eighty experiments are represented involving ten separate clutches of eggs.

It is apparent from this figure that embryonic squid are extremely resistant to cyanide, since at Stage X all survive 20 hours exposure to the N/1000 solution. In fact, according to a number of observations made by Barnes (1939, personal communication), segmenting eggs and embryos younger than Stage X are still more resistant. It must be pointed out, however, that the development subsequent to these long exposures is accompanied by a certain amount of deformity, so that perfectly normal ontogeny under these circumstances is unusual.

As development proceeds, the squid becomes less and less able to withstand the influence of cyanide, a fact which undoubtedly reflects a declining capacity for tolerating oxygen lack. A day or two after hatching death results from an exposure to cyanide of about one hour; adults however cannot survive an immersion of one minute. In Figure 1 a line has been drawn to indicate in an approximate fashion the time course of the change in viability with age. From the data shown in Figure 1 the longest exposure to cyanide which can be tolerated without death occurring has been estimated for the different developmental stages. These threshold durations are given in Table II.

TABLE II

Stage of development	Threshold-duration in N/1000 NaCN
X–XVI	20 hours
XVII	17 hours
XVIII	7 hours
Hatching	ca. 2 hours
1-day larva	ca. 1 hour
2-day larva	ca. ½ hour
4-day larva	ca. ¼ hour
Half-inch squid and adults	1 minute

It was noted in the experiments with 4-day larvae that the hearts continued to function even after the organism had been exposed to cyanide for an hour, an exposure which is, of course, fatal. A similar relative insensitivity of the heart to oxygen lack was reported by Redfield and Goodkind (1929). They found in adult squid asphyxiated by raising the carbon dioxide content of the water respired, that an hour after respiratory movements had ceased the heart was still beating.

Clearly, death from oxygen lack is not due in the first instance to any functional impairment of the circulatory system.

In contrast both the respiratory and locomotory movements of the mantle and siphon, which disappear very quickly in the adult upon exposure to cyanide, also fail to return to any appreciable extent after poisoning by cyanide. Thus not only does cyanide, and presumably oxygen lack, very quickly interfere with the function of the central nervous system, but these effects show less reversibility than do those of, for example, the circulatory system. This may perhaps be taken to indicate that the essential basis of the sensitivity to anoxia resides in the nervous system.

B. Effect of carbon dioxide

Redfield and Goodkind (1929) have shown that in the adult squid relatively low partial pressures of carbon dioxide prevent the haemocyanin of the blood from exercising its normal function in oxygen transport. Subjection of adult squid to partial pressures of carbon dioxide thus creates asphyxial conditions and brings about death from oxygen want. The respiratory function of the blood is quite completely eliminated, according to these investigators, by carbon dioxide partial pressures of 30–50 mm. Hg. We have compared therefore, in a few experiments, the reactions of adult and immature squid to carbon dioxide partial pressures in that range. The exact conditions employed and the observations which were made are summarized in Table III. These data show that at least until two days after hatch-

TABLE III

Stage	Temp. °C.	Av. p CO_2 mm.	Duration of exposure minutes	Result
XIV	24.3	40.0	30	Complete recovery
XVII–XVIII	23.3	48.3	30	Complete recovery
Hatching	24	37.1	31	65% recovery
Hatching	23.3	48.3	30	11% recovery
Hatched 1 day	23.4	45.0	19	Complete recovery
Hatched 1–2 days	23.4	45.0	19	Complete recovery
Hatched 1–2 days	23.3	48.3	30	No recovery
Hatched 2 days	23.4	45.0	19	Complete recovery
Adult (2)	20–25	26.2	16	No recovery
Adult (1)	24	37.1	12½	No recovery
Adult (2)	21.5	40.0	28	No recovery
Adult (1)	23.4	45.0	19	No recovery
Adult (1)	23.3	48.3	37	No recovery

ing *L. pealei* is quite unaffected by a 20-minute exposure to a carbon dioxide pressure as high as 45 mm. Hg. It is also very apparent that such an exposure is fatal to adults. However, larvae 1–2 days old fail to withstand a 48 mm. partial pressure of carbon dioxide for 30 minutes. Even this treatment does not affect younger (Stage XVIII) larvae. Confirming Redfield and Goodkind we find that adults show no recovery whatever after an exposure to a 26 mm. partial pressure of carbon dioxide for 15 minutes.

It is apparent from these data that resistance to carbon dioxide roughly parallels sensitivity to cyanide, and it seems probable therefore that carbon dioxide is in fact producing asphyxia just as does cyanide. Furthermore it must be concluded that in all the stages examined, the normal carriage of oxygen to the tissues of the

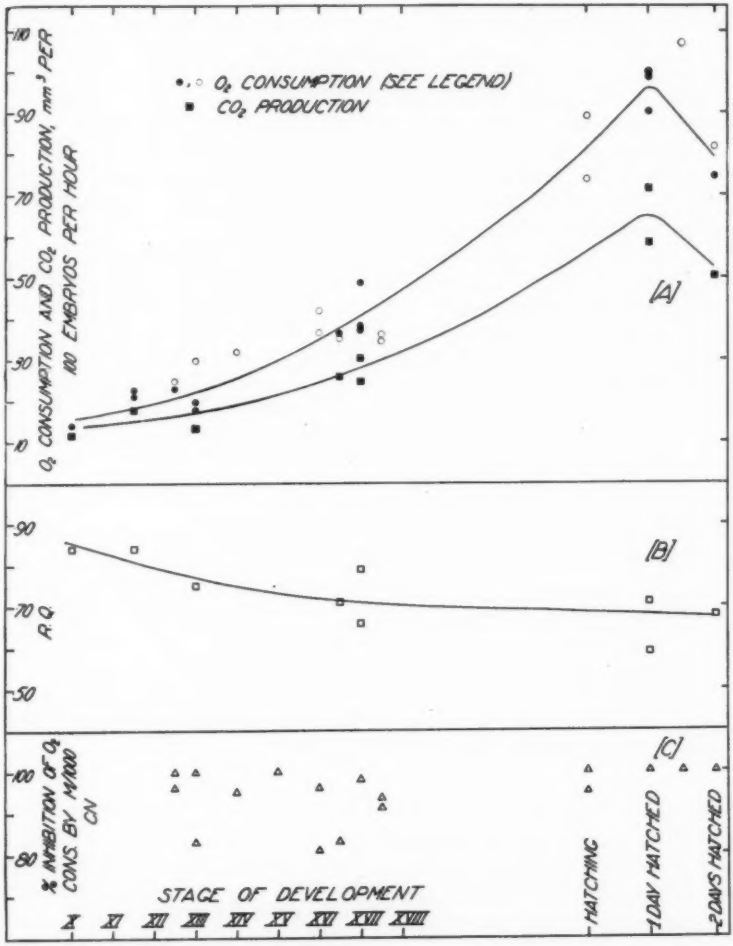

FIGURE 2. The rates of oxygen consumption and of carbon dioxide production, together with the respiratory quotient and the cyanide sensitivity, at different stages of development.

organism requires the intervention of haemocyanin as a carrier of oxygen. The respiratory function of the blood pigment thus appears to be well established at a time when resistance to cyanide (asphyxia) is still relatively high.

C. Respiration

The results of a series of experiments in which the normal rate of oxygen consumption of squid was determined at various stages of development are represented graphically in Figure 2a and b. Over the period examined a four- to eight-fold increase in the rate of oxygen consumption occurred. It is apparent from Figure 1 and Table II that over the same period the resistance to CN changed over forty-fold. While in general, then, the rate of oxygen consumption rises as the tolerance to asphyxia decreases, the latter actually changes 5–10 times as much as the former.

The drop in respiratory rate suggested shortly after hatching (Figure 2a), if real, may be associated with the rapid utilization of the remaining yolk as a result of increased activity of the organism and the failure to obtain natural food. Very little is known of the preferred food of squid larvae (Portmann and Bidder, 1928). In spite of the presence of abundant plankton, in our experience they survived only 4–5 days after hatching, dying apparently from starvation.

When squid embryos were exposed to cyanide in the respirometers, the respiration was observed to decline almost instantly (within a few minutes), indicating the immediate penetration of cyanide into the eggs. The rates of oxygen consumption in cyanide at the various stages are indicated in Figure 2c, from which figure it is evident that, in general, a high degree of inhibition (approximately 95 per cent) persists throughout development. It seems doubtful whether any significance can be attached to the apparent drop in sensitivity at Stages XVI–XVII. As far as the utilization of oxygen is concerned, therefore, there appears to be no evidence of a change in relative cyanide-sensitivity with age. It may be concluded that viability in this organism does not appear to be associated in any way with the possession of a cyanide-stable oxygen consumption.

Upon nine occasions, distributed over various stages of development, the normal rate of carbon dioxide production was also determined. From measurements of normal oxygen consumption made at the same time, the respective R.Q.'s were calculated and will be found in Figure 2b. The values show a slight tendency to rise during the early stages but there are no large fluctuations. This indicates that, as might be expected, the rate of carbon dioxide production rises throughout development just as does the rate of oxygen consumption. No marked changes in R.Q. are evident and there are certainly no changes which might be associated with the observed changes in viability on exposure to cyanide.

Discussion

The fact that the young of certain species are much better able to tolerate asphyxia than are the adults, has been known for a very long time as Johlin (1942) has recently emphasized. The newer work, which has already been referred to deals almost entirely with several of the common mammals. It might leave the impression that the decreasing tolerance of asphyxia seen in these during the few weeks following birth is a peculiarity of higher forms. Actually, this is apparently not the case, for the present work on squid indicates that in this species as well, young embryos typically survive exposures to asphyxia which are fatal to older embryos and to adults. Furthermore, the change in viability with development does not appear to be confined to any particular phase of that process, but instead takes place

gradually from the earliest stages examined. It will be shown elsewhere that an exactly similar change in viability with development occurs in several species of fish, and in frogs, so that in all probability it is quite a general phenomenon.

It is of course altogether likely that energy normally supplied by aerobic processes is replaced under asphyxial conditions by energy from anaerobic processes. The observations of Himwich, Bernstein, Herrlich, Chesler and Fazekas (1941) are particularly suggestive in this connection. These investigators report that the survival of newborn rats placed in an atmosphere of nitrogen is significantly shortened if the animals are also treated with iodoacetate. One might conclude that viability depends upon some particular cells and that the anaerobic capabilities of these become less as development proceeds. A priori however, it is just as logical to suppose that early in development viability depends on cells whose anaerobic capabilities are great, while later it comes to depend on the functioning of some more recently differentiated cells whose anaerobic capabilities are very much less. Supporting this latter conclusion are the facts (1) that the lethal effects of asphyxia are almost certainly exerted within the nervous system (Kabat and Schadewald, 1941; and Weinberger, Gibbon and Gibbon, 1940); (2) that the cells of the nervous system differ in their resistance to asphyxia (Kabat and Schadewald, 1941; Weinberger, Gibbon and Gibbon, 1940; van Harreveld, 1944); and (3) that in the mammal, at least, differentiation in the nervous system is taking place during those post partum stages in which the ability to survive asphyxia shows marked changes (Himwich and Fazekas, 1941).

Summary and Conclusions

1. The exposure to 0.001 N cyanide, which is fatal, gradually shortens from more than 20 hours in early embryos to 15 minutes in organisms examined four days after hatching. It is less than one minute in adults.

2. Cyanide brings about at least a 95 per cent inhibition of the rate of oxygen consumption of squid embryos at all stages examined. This inhibition develops immediately upon the addition of cyanide to the organisms.

3. A few data were obtained relating to the ability of squid embryos and adults to withstand increased partial pressures of carbon dioxide. It was found that this ability decreases as the embryos become older. The effect of the carbon dioxide is almost certainly to make the haemocyanin of the blood incapable of carrying oxygen. It is concluded that at all stages examined the normal rate of oxygen consumption requires the participation of the respiratory pigment of the circulation.

4. It may be inferred from these data on the effects of cyanide and of carbon dioxide that embryos are much more resistant to lack of oxygen than are adults. This general situation has been reported for certain other organisms. In the case of squid it is now evident that this change in resistance to asphyxia is a gradual process which is not related to any particular phase of development.

5. It is pointed out that it is undoubtedly damage to the central nervous system which is the immediate cause of death from oxygen lack, and that the site of the change in sensitivity to oxygen lack must therefore be sought in the central nervous system.

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CARBONIC ANHYDRASE IN MOLLUSCS[1]

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The catalytic action of carbonic anhydrase in the hydration of carbon dioxide suggests the possible role of this enzyme in the deposition of carbonate in shell (Meldrum and Roughton, 1933). This relationship has received consideration with respect to shell formation in birds in which it has been found that the shell-forming tissues contain carbonic anhydrase and inhibitors of that enzyme cause deficient shell formation (Common, 1941; Benesch, Barron and Mawson, 1944; Gutawska and Pozzani, 1945).

In molluscs the $CaCO_3$ of the shell is presumably formed first in mantle tissue with at least a portion of the carbonate having its origin in metabolic carbon dioxide (Robertson, 1941). The influence of carbonic anhydrase on the conversion of carbon dioxide to carbonate in molluscs has not been studied experimentally, though the enzyme has been reported in mantle tissue of a few pelecypods (Florkin and de Marchin, 1941; Maetz, 1946) as well as in other tissues of molluscs (van Goor, 1937; Sobotka and Kann, 1941). As an approach to this problem the carbonic anhydrase content of mantle tissues has been examined in 20 species of molluscs.

Methods

Tissues were blotted, weighed and ground thoroughly with a closely fitting mortar and pestle. Distilled water equivalent to 50 times the tissue weight was added and the suspension extracted 12 to 24 hours below 5° C. (Ferguson et al., 1937). Body fluids underwent similar storage without dilution. Extracted suspensions were agitated vigorously and pipetted directly without previous centrifugation. In one set of experiments tissues were blotted, placed in vials and kept frozen for several days. They were then extracted as previously described. Enzyme values for these tissues were similar to those for freshly extracted material.

The hydration of CO_2 in mantle tissue undoubtedly occurs in the presence of both carbonate and bicarbonate; and the colorimetric method (Brinkman, 1933; Leiner, 1938) used in the present study in which CO_2 is added to a carbonate-bicarbonate solution is appropriate for the estimation of carbonic anhydrase activity in this tissue. However, the values so obtained will include the specific action of the buffer and indicator on the enzyme (Roughton and Booth, 1946). The endpoint was determined by means of a comparison tube containing phenol red buffered at pH 7.35, a value suitable both from the standpoint of rapidity of color change and rate of enzyme action. The activity of tissue extracts was measured at a final concentration of 1 : 500 and body fluids at 1 : 10. Two types of controls were used. In one the time required for reading the endpoint was determined with distilled water substituted for the extract, and in the other a portion of the extract was heated in

[1] Aided by a grant from the Duke University Research Council.

TABLE I

Species	Ratio I: $\frac{\text{No extract}}{\text{Extract}}$	Ratio II: $\frac{\text{Heated extract}}{\text{Extract}}$
Pelecypoda:		
Atrina rigida		
mantle edge	0.8; 1.0; 0.9; 1.0; 1.0	1.1; 1.1; — — —
mantle without edge	0.7; 1.0; 0.9; 1.0; 0.9	1.0; 1.1; — — —
pericardial fluid	0.6; 0.9; 0.7; 0.6; 0.9*	1.2; 1.1; — — —
gill	2.8; —; 2.2; 3.4; —	4.2; —; — — —
adductor muscle	—; 0.8; 1.5; 1.2; —	—; 0.6; — — —
Venus mercenaria		
mantle edge	2.6; 4.8; 2.5;	3.4; 7.2;
mantle without edge	3.3; —; 2.6; 4.1	4.8; —
mantle cavity fluid	0.8; 1.4; 2.0;	1.5; 3.5;
Ostrea virginica		
whole mantle	4.9; 1.5; 1.8; 3.6;	7.2; 2.2
general body fluid	7.1; 2.2; —; —;	
gill	2.8; —; 5.5; —;	3.7;
adductor muscle	—; —; 1.0; —;	
Macrocallista nimbosa		
mantle edge	1.2; 1.0; 1.5; 1.9	2.0; 1.4;
mantle without edge	1.8; 2.1; 1.6;	3.0; 3.1;
mantle cavity fluid	0.6;	1.5;
gill	5.0	
adductor muscle	1.2;	
Dosinia discus		
mantle edge	1.1; 1.9; 2.1;	1.4;
mantle without edge	2.0; 2.3; 2.5;	3.1;
body fluid	1.5; 14.0*; 1.1*;	3.9;
adductor muscle	10.0; 6.4	—; —; —; 4.0
Elliptio complanatus		
mantle edge	2.5; 2.0;	2.6; 2.1;
mantle without edge	2.8; 2.5;	3.0; 2.6;
mantle cavity fluid	4.7; 1.7;	5.1; 2.0;
adductor muscle	3.0; 3.7;	2.4; 4.1;
Tagelus gibbus		
mantle edge	2.7; 2.1;	
mantle without edge	3.4; 4.2;	
mantle cavity fluid	2.7; 1.6;	
gill	5.0;	
Pecten irradians		
whole mantle	2.5; —; 2.0	
mantle edge	5.2; 2.4; —	
pericardial fluid	—; —; 1.4	
Divaricella quadrisulcata		
whole mantle*	3.2	
Ensis directis		
mantle edge	3.4	
mantle without edge	8.1	
Modiolus tulipus		
whole mantle	2.0	
Cardium muricatum		
whole mantle	4.3	

TABLE I—*Continued*

Species	Ratio I: $\frac{\text{No extract}}{\text{Extract}}$	Ratio II: $\frac{\text{Heated extract}}{\text{Extract}}$
Gastropoda:		
Busycon carica		
whole mantle	1.2; 0.8; 1.6; 1.1; 3.0; 2.1	1.7
blood	0.6; —; 0.8; 0.6;	1.9
gill	1.8;	
Viviparus japonicus		
whole mantle	1.4; 1.6	1.9; 2.2
mantle cavity fluid	0.6; 0.9	—; 1.0
Crepidula fornicata		
whole mantle	0.4; 0.6; 1.2	
mantle cavity fluid	0.9;	
Fasciolaria distans		
whole mantle	2.4; 5.1;	
blood	2.3;	
Anomia simplex		
whole mantle*	3.6	
Polynices duplicata		
whole mantle	2.7	
Sinum perspectivum		
whole mantle	2.8	
Diodora alternata		
whole mantle	2.0	

* Sample composite of tissues from several individuals.

boiling water for 5 minutes. The activity is expressed as the ratio of the time of reaction in the absence and presence of the extract and also as the ratio of time of reaction for heated and unheated extracts. Duplicate determinations were made in a large proportion of the cases and the average taken.

We are indebted to Mr. W. H. Sutcliffe, Jr., for aid in the collection of material and to Dr. C. G. Bookhout and Dr. H. van der Schalie for its identification.

RESULTS

Each figure in Table I represents the relative catalytic action of a single sample. Tissues from a particular individual are grouped in columns. In those cases in which both ratios were determined for the tissue the figures stand in corresponding positions under the two ratio headings.

It is evident that most of the tissues examined catalyzed the hydration of CO_2. Considered from the standpoint of Ratio I the outstanding exceptions are certain tissues of *Atrina rigida, Macrocallista nimbosa, Busycon carica, Crepidula fornicata,* and *Viviparus japonicus.* However, several of these instances of the absence of catalysis are only apparent as shown by comparing heated and unheated extracts (Ratio II). Of the individuals examined, carbonic anhydrase activity under the conditions of measurement is negligible or absent only in the mantle and pericardial fluid of *Atrina rigida,* the mantle-shell cavity fluid of *Viviparus japonicus,* and perhaps also in the mantle and mantle-shell cavity fluid of *Crepidula fornicata.*

The higher values for Ratio II result from the slowing of the uncatalyzed reaction by the heated extract and are probably due to the presence of carbonate and other substances.[2] Figures given by Maetz (1946) show an analogous effect. Because of this effect the values (Ratio I) of all tissues for which data on heated extracts are not available may be considered minimal except for muscle. The four adductor muscles examined showed an opposite effect and one which would be expected with acid production.

Carbonic anhydrase activity was demonstrated in the body fluids of nearly all forms studied and in the blood of two species of gastropods. Florkin (1935) and van Goor (1937) investigated blood of a large number of species and found no evidence of the enzyme. This may have been the result of the high dilutions employed.

Discussion

The presence of carbonic anhydrase in the mantle tissues of most of the molluscs examined indicates the possibility that this enzyme plays a part in the formation of carbonate. Both the mantle edge and the body of the mantle contain the enzyme and both are concerned with the deposition of carbonate (Robertson, 1941). Of course the presence of the enzyme cannot be taken as evidence that normal shell formation is dependent upon its activity, and its presence in other tissues suggests additional functions. The negligible activity in *Atrina rigida* and *Crepidula fornicata* suggests that certain forms may, in fact, lay down shell in its absence. In this connection a study of the rate of shell formation in the presence of inhibitors would be suggestive, provided the inhibitors did not interfere with the growth of other structures. The catalytic action of carbonic anhydrase may be expected to assume increased importance as the temperature is decreased, especially in forms such as the oyster in which shell formation proceeds during hibernation at temperatures below 4° C. (Galtsoff, 1934).

Summary

Carbonic anhydrase has been demonstrated in the mantle tissues and body fluids of most of 12 species of pelecypods and 8 species of gastropods. Its presence suggests the possibility of importance in shell formation in most species but negligible activity in some indicates they may deposit shell in its absence.

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[2] This effect is probably complex and may involve: (1) a change in buffer action, (2) the effect of specific substances on the enzyme, (3) an alteration of the initial pH, and (4) modification of the properties of the extract in a manner other than in destruction of the enzyme.

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PHYSIOLOGY OF INSECT DIAPAUSE. III. THE PROTHORACIC GLANDS IN THE CECROPIA SILKWORM, WITH SPECIAL REFERENCE TO THEIR SIGNIFICANCE IN EMBRYONIC AND POSTEMBRYONIC DEVELOPMENT

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In recent years it has become increasingly evident that insect metamorphosis is under the control of an array of factors generally presumed to be hormonal in character. Interest has heretofore centered around the endocrine functions of the brain, the corpora cardiaca, and the corpora allata, including the "ring gland" of Diptera. Since these organs are usually situated in the head, it has not been easy to account for the fact that a thoracic center exercises important control over the embryonic and postembryonic development of many species (Richards and Miller, 1937; Richards, 1937).

The existence of a thoracic "differentiation center" seems, indeed, to be a distinctive feature of the embryology of all insects (Schnetter, 1934). As will become evident subsequently, the position of this embryonic center is of special interest. According to Richards and Miller (page 35), it "extends from the second gnathal (maxillary) segment to the second thoracic segment with its midpoint in the anterior half of the presumptive prothorax."

In the case of postembryonic development the evidence in favor of the existence of a thoracic "differentiation center" has been especially prominent in studies of the Lepidoptera. Here the thorax is believed to preside over moulting (Fukuda, 1940b), pupation (Bodenstein, 1938; Bounhiol, 1938; Fukuda, 1940a), and adult development (Hachlow, 1931; Bounhiol, 1938; Fukuda, 1941; Williams, 1947). The thorax has not been implicated, to date, in the postembryonic development of the Diptera, Orthoptera, or Hemiptera, but according to Geigy and Ochsé (1940) the thorax or first abdominal segment controls pupation in the megalopteran, *Sialis*.

These observations take on renewed interest as a result of Fukuda's studies of the commercial silkworm, *Bombyx mori*, where pupation and adult formation were found to be dependent on the function of a previously overlooked organ, the "prothoracic glands." For the first time the identity of a thoracic differentiation center was thereby established. The prothoracic glands therefore merit special attention, for homologous structures may be widely distributed among insects and conceivably act as the thoracic center that is known to control embryonic and, in certain species, postembryonic development. For this reason the existing literature pertaining to the prothoracic glands will be briefly reviewed.

Prothoracic glands were first described by Toyama (1902) in his study of the embryology of *Bombyx mori*. They arise early in embryonic development as

[1] The assistance of the Lalor Foundation of Wilmington, Delaware, is gratefully acknowledged.

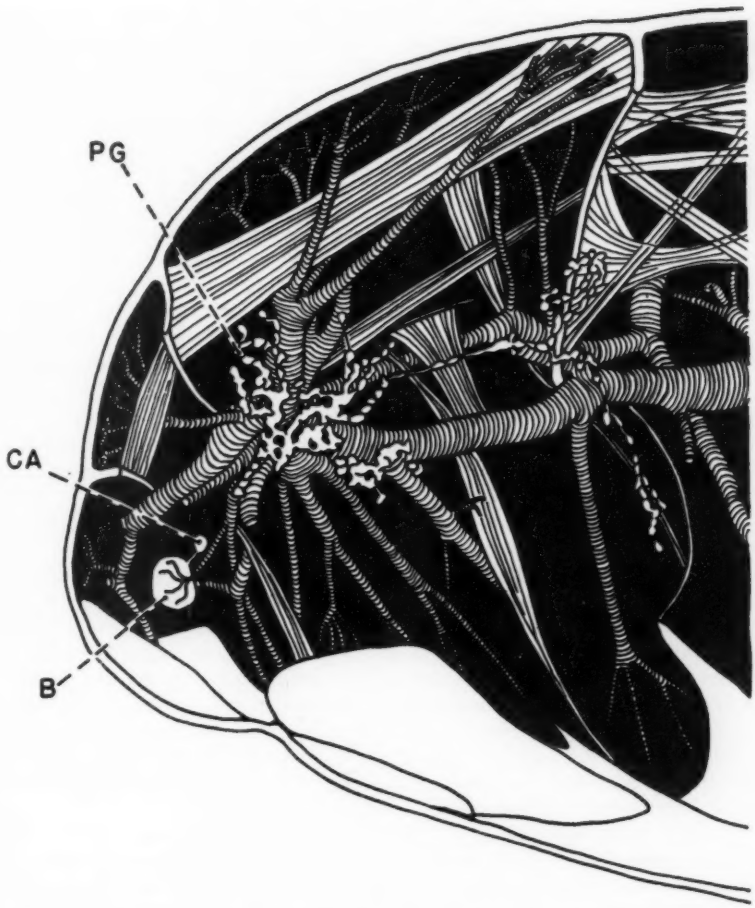

FIGURE 1. The prothoracic gland in the right half of the thorax of a diapausing Cecropia pupa. Fat body and numerous other tissues have been cleared away: B, Brain; CA, Corpus allatum and Corpus cardiacum; PG, Prothoracic glands.

epithelial invaginations of the lateral part of the second maxillary segment, from which they extend posteriorly into the prothorax. This location may, to advantage, be compared with that of the "differentiation center" in embryonic insects, described above.

Although Toyama's discovery has been generally overlooked, it is to his credit that he recognized the prothoracic glands not only as being hitherto unknown, but also as being glandular organs. They were named "hypostigmatic glands," an unfortunate designation, since the term had previously been applied to wholly different

tissues in the Lepidoptera (Verson and Bisson, 1891). Twenty-eight years elapsed before the glands were next mentioned in the literature. Ke (1930) then named the organs "prothoracic glands" and described certain variations in their branching. In a paper to be published in the near future, Lee (1948) will present a morphological study of the prothoracic glands in lepidopterous larvae, with special reference to their innervation.

Physiological function for the prothoracic glands was first discovered by Fukuda (1940a, 1940b, 1941) in a series of studies reviewed elsewhere (Williams, 1948). Most recently, the function of the prothoracic glands has been considered in relation to adult development of several species of silkworms (Williams, 1947, 1948).

Morphology and Histology

In my experience the prothoracic glands can be demonstrated most satisfactorily by gross dissections of fresh material submerged in insect Ringer's solution. Even under these circumstances the organs are not easy to find on account of their transparency. This difficulty may be minimized by vitally staining the preparation with methylene blue, a treatment that stains the prothoracic glands more or less intensely without impairing their viability in physiological experiments.

The following description is based on numerous dissections of the Cecropia silkworm and especially of the diapausing pupa of this species:

As shown in Figure 1, the prothoracic glands are bilaterally placed in the pro- and mesothorax. Each gland consists of a main mass and a number of branches. The main mass lies immediately internal to the large tracheae at the level of the prothoracic spiracle. Branches radiate from this main mass, as indicated in Figure 1. The branch extending in the direction of the brain is short and compact, but the other branches consist, for the most part, of fusiform or stellate cells strung together on tenuous, intercellular bridges. In the region of the mesothoracic spiracle the cells of the gland show a second concentration. All of these branches are laterally placed in the insect and are covered and partially entwined with fat-body. The gland is richly supplied with nerves and tracheoles.

The microscopic structure of the gland is indicated in Figures 2 and 3. Little internal structure is visible in the living gland (Fig. 2) and greater detail is not obtained after fixation and staining with most agents. In my experience, best results are obtained by fixation in Helly's solution and staining with haematoxylin. As shown in Figure 3, the gland is then found to consist of enormous, complexly folded nuclei surrounded by a scanty, cytoplasmic syncytium. Each nucleus contains a single, prominent nucleolus.

Although the above description applies, specifically, to the diapausing pupa, the prothoracic glands show essentially the same arrangement in the later larval instars. With the onset of adult development, dissection becomes very difficult, but the glands are still present and seem to assume a more racemose configuration in the region of the prothoracic spiracles. I have not succeeded in demonstrating the organs in the adult and believe them to be absent. It is probable that a description of the glands in young larvae, in developing pupae, and in the adult (?) must await reconstruction from serial sections.

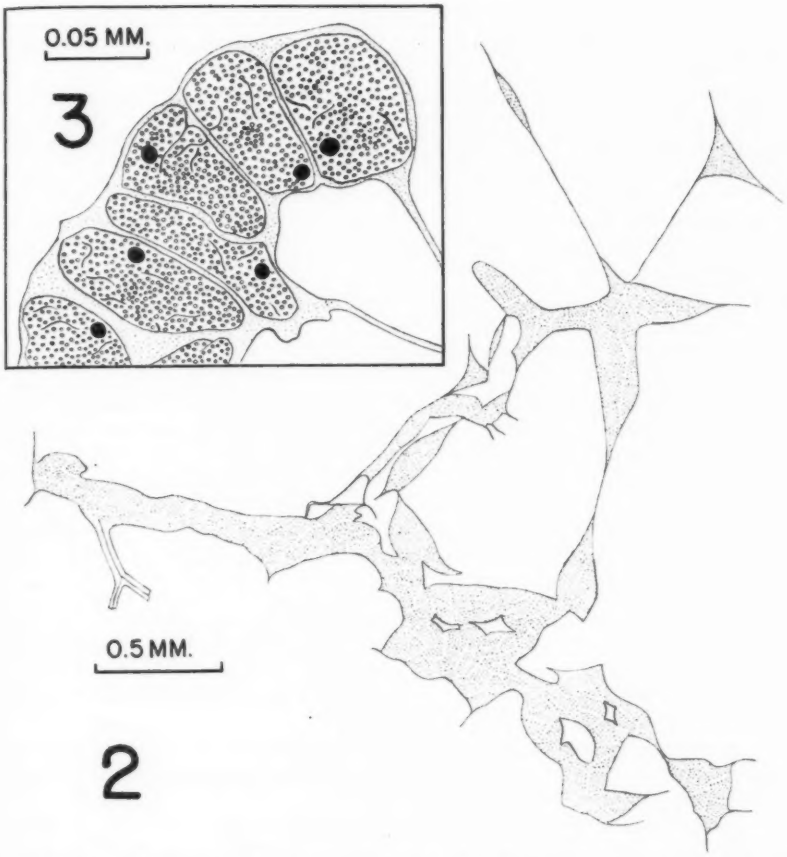

FIGURE 2. Living fragment of a prothoracic gland of a diapausing Cecropia pupa, showing branching and anastomosing structure

FIGURE 3. Highly magnified view of a fragment of a prothoracic gland of a diapausing Cecropia pupa. Fixed in Helly's solution and stained with Delafield's haematoxylin.

DISCUSSION

It is not difficult to understand why prothoracic glands were overlooked for so many years. For, in the species that I have examined, the gland is a tissue rather than a compact organ. I regard complete or even partial excision of the prothoracic glands as technically incompatible with the survival of the animal. To secure individuals devoid of prothoracic glands, one must necessarily work with isolated abdomens.

Prothoracic glands are widely distributed among the Lepidoptera and, most probably, occur in all members of the Order (Lee, 1948). Preliminary, gross dis-

section has failed to reveal their presence in larvae of Diptera, Coleoptera, and Trichoptera, but they are conspicuous in the hymenopterous larva of the saw-fly, *Cimbex americana*.

Of special interest is the description by Pflugfelder (1938) of a bilateral glandular cell complex in the head region of the walking-stick, *Dixippus*. This complex was termed the "ventral head gland of inner secretion." It arises as an epithelial invagination in the region of the head and apparently extends into the prothorax where it is attached to the muscles and tracheae of this region. It attains maximal size just before the last moult, at which time the nuclei become lobulated. After the last moult the nuclear membrane is lost and the chromatin lies irregularly in the cytoplasm. The gland is degenerate in the adult. Pflugfelder suggested that the gland might play a role in nymphal development, but considered its extirpation to be impossible.

On the basis of this description, I propose that the ventral head glands of Pflugfelder are homologous with the prothoracic glands of Lepidoptera. If this is confirmed, then prothoracic glands must be present in certain hemimetabolous as well as holometabolous insects and the endocrinology of insect development must be re-examined from this point of view.

A careful survey of all Orders is required. Since the gland may conceivably vary in its structure and position, such a search should be directed towards a study of embryos with special reference to the presence and fate of invaginations of the lateral part of the second maxillary segment.

Summary

1. The data relative to the thoracic control of insect development are surveyed. Embryonic development seems to be controlled by a thoracic center in all insects. Postembryonic development seems to be controlled by a thoracic center in at least the Lepidoptera and the Megaloptera.

2. In the Lepidoptera the thoracic center for postembryonic development can be identified as the "prothoracic gland." A review is presented of the literature describing these organs.

3. The thoracic center for embryonic development coincides with the position occupied by the prothoracic glands in lepidopterous embryos. It is therefore possible that the prothoracic glands may function as the embryonic differentiation center in at least certain species.

4. The morphology and histology of the prothoracic glands are described in the Cecropia silkworm. Homologous organs are present in saw-fly larvae and most probably in the walking-stick, *Dixippus*.

5. Since prothoracic glands, or their homologues, may be widely distributed in embryonic and postembryonic stages of insects, these organs present special physiological interest and merit further study.

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THE RELATION OF TEMPERATURE TO OXYGEN CONSUMPTION IN THE GOLDFISH [1]

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Introduction

In general, studies on the relation of the metabolic rate to temperature have been limited to a consideration of the standard metabolism. Standard metabolism in animals has been taken as the approximate equivalent to basal metabolism in man. A distinction is made in deference to the fact that animals cannot approach the experiment in such a consciously basal state as can the human subject. However, from the ecological point of view, which recognizes that much of the measure of success which an animal may enjoy is due to its ability to be active, it is not enough to know the basal or standard metabolic state alone. It is of equal importance to measure the maximum metabolic rate, unless changes in the maximum rate follow no rule, or can be predicted from the standard rate. For fish at least there appear to be no data which establish either of these two provisos. For these reasons we undertook to explore the relation between a standard and a maximum level of metabolism in the goldfish and the ambient temperature. The two levels chosen were (1) a standard which was the minimum resting level in the diurnal cycle, a level as near basal as we could establish, and (2) a "maximum" which was the rate which appeared to be the highest the fish were able to maintain continuously until fatigue set in.

Material and Methods

The goldfish used were a single lot of animals approaching one year old with an average weight of 3.8 gms. These fish were similar in age and source to those used for the exploration of various other physiological characteristics of the species (Fry, Brett and Clawson, 1942; Brett, 1946; Fry, Black and Black, 1947; and Fry and Hart, 1947). They were stored in running water and fed on a commercial fox food. Previous to any experiment the fish were acclimated to the particular temperature at which the experiment was to be carried out by maintaining them at that temperature for a length of time that would enable them to come to a stable state, as far as this is indicated by the stability of the upper lethal temperature (Brett, 1946). This period ranged from twenty days at 5° C. to less than four at 35° C. During this acclimation period the fish fed well. Both the standard and maximum levels of metabolism for any given temperature were measured on the same subjects, but the measurements at different temperatures were carried out on different samples drawn from the common stock. The metabolic rates were measured by determining the rate of oxygen consumption.

[1] An abstract of this paper appeared under the same title in the *Anatonomical Record*, Vol. 96, No. 4 Supp., December, 1946.

All the rates of oxygen uptake were determined by means of the unmodified Winkler method. While nitrite contamination may introduce error into oxygen determinations (Allee and Oesting, 1934) it does not seem to have been a serious factor here. A number of checks with the Rideal-Stewart modification gave essentially the same readings as the unmodified Winkler's. Moreover, consistent results were obtained on check runs in which the initial levels of oxygen differed and hence similar levels were reached after different intervals of time.

Both the standard and the maximum levels of oxygen consumption were measured by determining the fall in oxygen content in closed containers (Fig. 1). The respiration chamber used to determine the standard level was a two-liter Erlenmeyer flask. The flask was fitted with a two-hole stopper through which projected

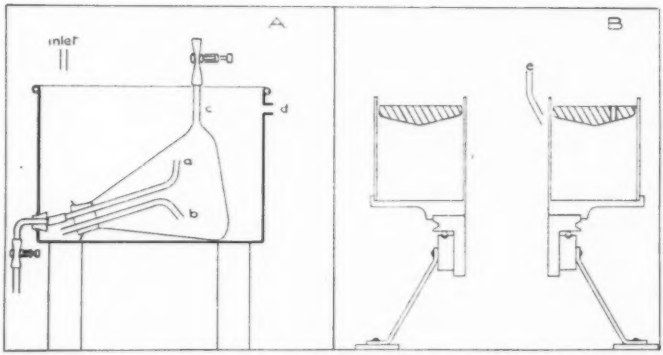

FIGURE 1. Arrangement of apparatus for measuring: A, standard metabolism; B, active metabolism. See text for detailed descriptions.

two glass tubes (a and b), the inner ends of which were bent at right angles and spread well apart. A glass side arm (c) was blown into the Erlenmeyer at is widest diameter. This side arm was provided with a length of rubber tubing and a pinch cock. The fish were placed in water in the flask which was then placed on its side in a water bath with the glass tube uppermost. Tube (a) was connected to a second tube which led through the wall of the bath. Tube (b) was open to the bath. A supply of water of the desired temperature was led into the bath and aerated vigrously. Excess water passed out an overflow (d), thus maintaining a constant level of water in the bath. Water from the bath passed by tube (b) through the Erlenmeyer as long as (a) was open. The side arm (c) collected any bubbles which formed in the flask. Normally, tube (a) was left open, and the flow through the Erlenmeyer was so adjusted as to be sufficient to make the difference in oxygen content in the inflowing and outflowing water negligible.

The chambers for measuring standard metabolism were set up in a basement room lighted by overhead lights in the daytime but dark at night, except for a shrouded fifty-watt bulb near the floor. The fish were in almost complete darkness at night since the metal bath shielded them from the light on the floor.

At temperatures of 20° C. and lower, twenty fish were placed in the Erlenmeyer; at higher temperatures the number was reduced to ten. The fish were placed in

the chambers on the day previous to the experiment, and water samples for oxygen determination were taken as described below over at least one complete twenty-four hour cycle. All samples were taken as quietly as possible in order to minimize any disturbance of the subjects. However, at that time we were not conversant with the findings of Spoor (1946), and we undoubtedly did not eliminate such disturbance entirely. The minimum oxygen consumption generally occurred between 10 P.M. and dawn. The titration figures for each twenty-four hour cycle were plotted, and the lowest point during the night hours found by interpolation. The standard rate of oxygen consumption was calculated from the value thus found.

When a measurement of the oxygen consumption was to be made, any bubbles in tube (c) were carefully drawn out by suction. Water samples were taken from tube (a) and the bath. Tube (a) was then closed by the screw cock, thus isolating the water in the flask except for the connection through the relatively long and narrow tube (b). The time interval between the first and second sample was dependent on the metabolic rate. These intervals varied from four hours at 5° C. to thirty minutes at 35° C. The time between the initial and final samples was so chosen that the oxygen tension in the chamber did not fall low enough to affect the rate of oxygen uptake, a number of preliminary experiments being performed to confirm this fact. After the appropriate interval of time a second sample of approximately 100 cc. was drawn from tube (a) into a 50 cc. sample bottle, the first 50 cc. being used to flush, the second 50 cc. being retained as the sample. This partitioning of the sample was accomplished by taking the time during the first filling of the bottle and then allowing the water to flow for a further equal time before removing the sample bottle. It was presumed that this relatively rapid withdrawal of 100 cc. took place without any contamination from the corresponding 100 cc. of water which entered the Erlenmeyer from the bath.

The maximum level of metabolism was measured on fish swimming steadily against a current generated in a rotating chamber (Fig. 1B). This chamber was an annular vessel of rectangular cross section of which the outer wall was glass and the inner wall and bottom metal. The outside diameter was 12 inches, the width 3 inches, and the depth 6 inches. This apparatus is an elaboration of that originally used by Black, Fry and Scott (1939). A measured volume of water was placed in the chamber, the fish introduced, and the surface of the water protected from the atmosphere by means of a loosely fitting cover. Samples of water for the determination of the oxygen content were obtained by withdrawing 100 cc. by pipette. The pipette was then inserted to the bottom of a 50 cc. sample bottle and approximately 75 cc. of the sample was delivered into it, the excess being flushed out. The last 25 cc. in the pipette were discarded. Since the cover did not isolate the water completely from the atmosphere, some exchange of gas took place between the water and the air. The magnitude of this exchange at different temperatures and oxygen tensions was determined by filling the chamber with water which had been boiled and flushed with nitrogen, and then following the increase in oxygen with the cover in place. We are indebted to Miss J. M. Graham for making these determinations. A correction based on these results was applied to the apparent rates of oxygen uptake determined experimentally. The temperature of the water in the rotating chamber was controlled by playing a stream of water on its inner wall through tube (e).

Results

(*a*) *Relation of temperature to standard metabolism*

The relation of the standard metabolism of the goldfish to temperature has been investigated by other workers on a number of occasions. On two occasions in particular (Ege and Krogh, 1914; Gardner, King and Powers, 1922) this relation has been worked out over a considerable range of temperature. The results of these workers are displayed with our own in Figure 2. The logarithms of the

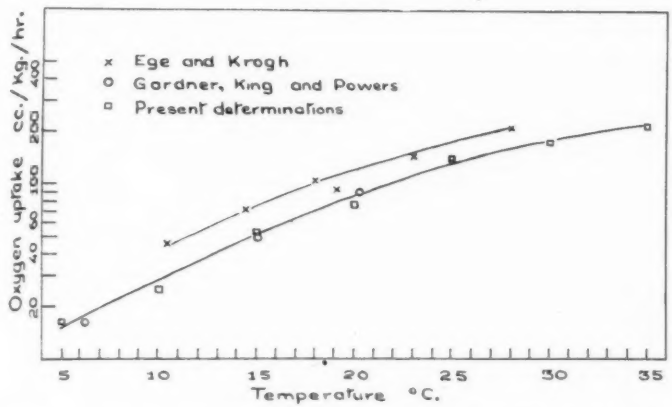

FIGURE 2. The relation of temperature to standard metabolism in the goldfish.

rates of oxygen uptake are used in this figure rather than the actual values so that a comparison can be made between the relative rates of change. The relative rates of change with increase in temperature are very similar in the three sets of data. Since different techniques were used in the three cases, such agreement can probably be taken as a satisfactory indication that the relative change is a stable characteristic of the species, although it must be remembered that this particular curve is characteristic of quite a number of other animals also (Krogh, 1941).

There does not appear to be any indication of any difference in this relative rate with difference in size such as Wells (1935) believed was the case in *Fundulus parvipinnis*. However, his evidence is somewhat confused by the fact that the rates at different temperatures were measured at different times of day. Moreover, a good deal of the divergence in the rate-temperature curves he gives is an attribute of the magnitude of the absolute rates and disappears if a semi-logarithmic plot is used, as in Figure 2.

The rate of oxygen uptake per unit weight does not bear any consistent relation with size in the three sets of data in Figure 2. Such disagreement is probably due to the fact that the standard states used by the three groups of investigators were not the same.

(b) Relation of temperature and oxygen tension to the maximum rate of oxygen uptake

So far as we are aware, no other workers have explored the effect of temperature on the maximum rate of oxygen uptake by the goldfish, nor have we examined this at all exhaustively, for we have confined our observations to what we believe to be the maximum level of oxygen uptake that can be maintained steadily.

The maximum rate was measured over a range of oxygen tensions down to those approaching asphyxial levels. In this way the effects of oxygen and temperature can be determined simultaneously and the scope of the experiment thus extended. The technique used was the familiar and simple one of allowing the organism to exhaust the oxygen in an isolated volume of water. In the case of goldfish it can probably be safely assumed that the small increase in carbon dioxide that results from the respiratory exchange in such experiments has no appreciable depressing effect on the rate of oxygen uptake. Under the conditions of our experiments the highest tension this respiratory carbon dioxide would reach in Toronto tap water is about 20 mm. at 35° C., a level which would appear to be far from any that would exert a limiting effect (Fry, Black and Black, 1947).

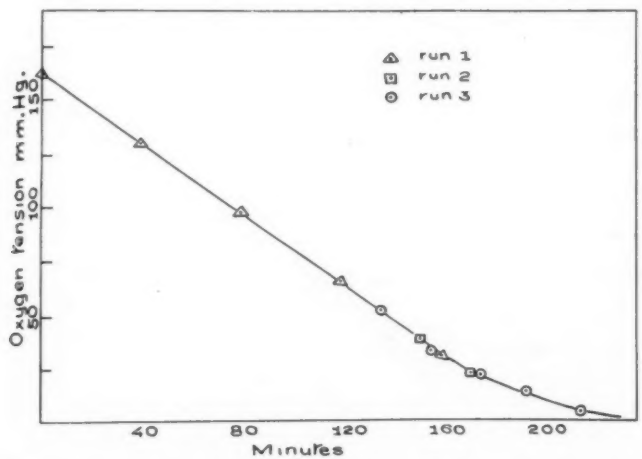

FIGURE 3. The time course of oxygen depletion in the rotating chamber, in a typical measurement of the metabolism of active goldfish (temperature 15° C., 20 fish, 4 litres water).

The course of the removal of oxygen from the water in the respiration chamber in a typical experiment is shown in Figure 3. Figure 3 presents the same picture as that found by Toryu (1927) for this species. Thus, our figures confirm his findings that the gold fish, at least when of the size of our subjects, has a respiratory system that is independent of the oxygen tension down to relatively low levels even under conditions of maximum continuous activity.

The maximum steady rates of oxygen uptake in relation to oxygen tension found at the different temperatures investigated are shown in Figure 4, where the rate of

oxygen uptake is plotted gainst oxygen tension. These curves were derived by taking tangents from the appropriate graphs similar to Figure 3. Owing to the low level at which the oxygen uptake becomes dependent on oxygen tension, the data on which the descending portions of these curves are based are rather meager and no precise accuracy is claimed for them such as was attained by Maloeuf (1937) in his investigations. In particular, the curve determined at 20° C. is not consistent with the trend of the remaining data. However, although the proportional error may be considerable, the absolute error must be of no great order since the range of tensions occupied by this portion of the curve is in all cases less than 40 mm.

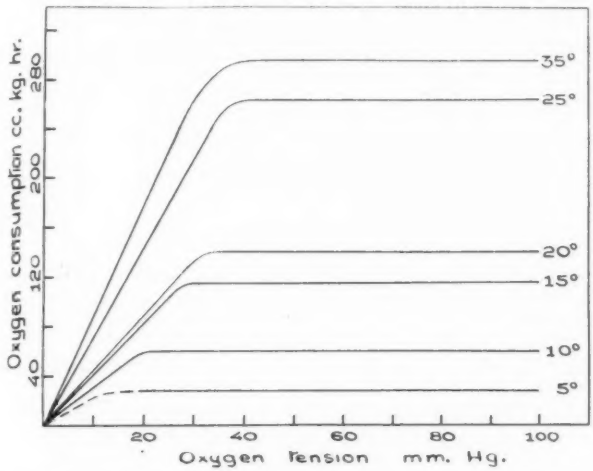

FIGURE 4. The relation of the maximum steady rate of oxygen consumption to oxygen tension at various temperatures.

While the physiological significance of the phenomenon commonly known as respiratory dependence has been often discussed at length since the beginning of the century, its ecological implications do not appear to ever have been clearly stated, except in connection with man in the case of the conquest of Mount Everest (Henderson, 1939).

The data in Figures 2 and 4 appear to us to offer two physiological indices that may be of great use to the ecologist. These have been designated by Fry (1947) as "the incipient limiting level" and "the level of no excess activity." The incipient limiting level is that level of oxygen tension below which the rate of oxygen uptake at the maximum steady state of activity begins to be reduced. This is the point of inflection of the curves in Figure 4. The incipient limiting levels of oxygen tension thus determined for the goldfish are given in Table I. The point of inflection in Toryu's data occurs at an oxygen tension of the order of these values, which appear to us to indicate that the respiratory rate he was measuring was well above the standard level under the conditions to which he was subjecting his animals. Such circumstances have been commonly remarked.

The level of no excess activity has been taken as that level of oxygen at which the animal can no longer satisfy more than its standard requirements for oxygen in spite of the utmost efforts of its ventilatory and circulatory apparatus. This level for the goldfish at a given temperature can be determined by finding on the curves in Figure 4 the tension corresponding to the oxygen consumption under standard conditions at the same temperature as determined from Figure 2. These values are presented in Table II.

TABLE I

Tensions of oxygen at various temperatures below which the maximum steady rate of oxygen uptake is reduced in young goldfish acclimated to the temperatures at which the measurements are made

Temperature °C.	Incipient limiting level of oxygen mm. Hg
5	15
10	21
15	29
20	34
25	39
35	40

TABLE II

Estimated oxygen tensions at which young goldfish can take up no more oxygen than will satisfy the requirements of standard metabolism in subjects acclimated to the temperature at which the estimate is made

The values for the standard metabolic rate in column 1 were taken from the smooth curve through our data in Figure 2.

Temperature °C.	Standard met. rate cc. Kg. hr.	Level of no excess activity mm. Hg	Asphyxial level[1] mm. Hg	Residual level[2] mm. Hg
1				4
2.6			nil	
5	8	4		
7				4
10	24	8		
11.4			8	
15	50	10		4
20	85	18		7
25	140	17		8
27.2			12	
32				8
35	225	25		

[1] Gardner and King, 1922.
[2] Fry, Black and Black, 1947.

The levels of no excess activity given in Table II have, of course, been abstracted from a purely experimental situation, and the worth of such data to the ecologist must ultimately depend on proof that they have real significance as values limiting the activity of the organism in nature. The greatest uncertainty lies in the assumption that the standard level chosen was a fair approximation of the minimal

respiratory requirements of the animal. We have not investigated the matter exhaustively, but a comparison is made in Table II with the values given by Gardner and King (1922) for the asphyxial level of oxygen for goldfish and what for convenience have been termed "residual levels" given by Fry, Black and Black (1947). These residual levels are the tensions of oxygen which remain in water in sealed bottles in which the fish have been allowed to exhaust the oxygen supply until they died. Thus, since some oxygen transport still goes on after the animals have been asphyxiated, and since the volume of water in which the fish were confined was small these residual values are somewhat below the asphyxial level. Taking the various circumstances into consideration, these three sets of data show fair agreement.

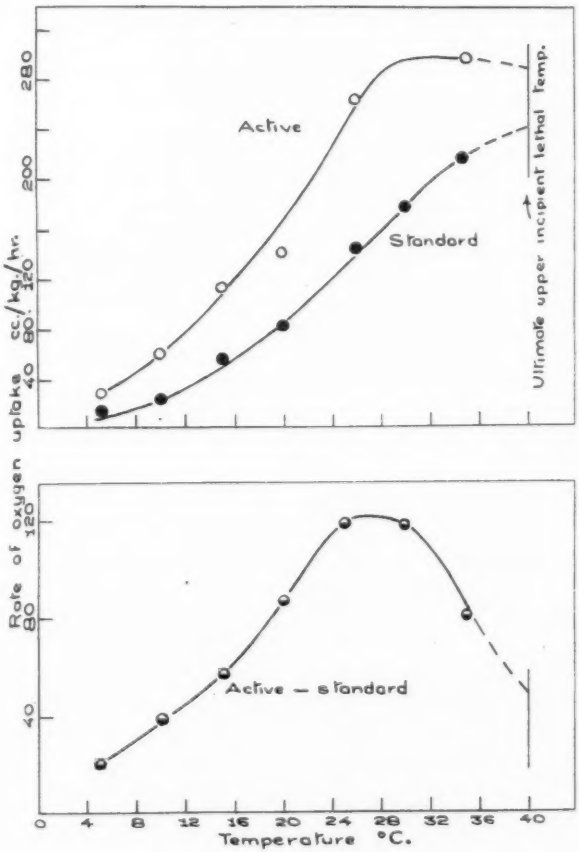

FIGURE 5. The relation between temperature and the active and standard levels of oxygen uptake.

(c) The difference between the maximum and standard levels of metabolism at various temperatures

Assuming that the standard level of metabolism as measured here is a reasonable approximation of the maintenance metabolism, then the metabolism available for activity will be of the order of the difference between the maximum and standard levels. It will not be the whole difference, since with increasing activity the cost of ventilation and circulation and the other functions necessary to allow the external work to be carried on will, of course, increase, and some of the difference will be consumed for the increase of these auxiliary activities. The relation between temperature and the maximum rate of oxygen uptake at levels where the oxygen tension is not limiting, that is, at levels along the horizontal portions of the curves in Figure 4, is shown, together with the curve for standard metabolism in Figure 5.

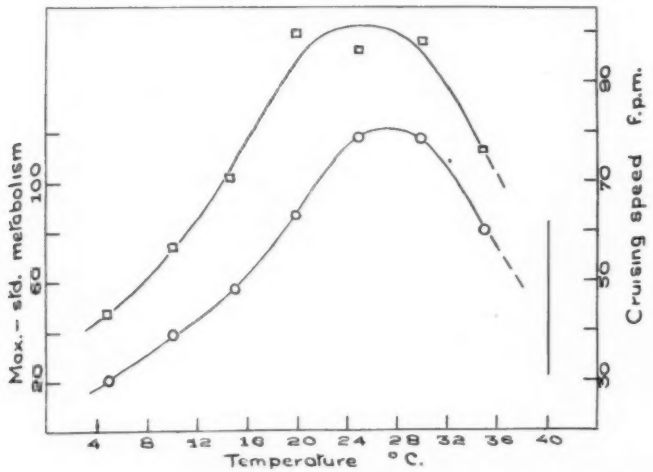

FIGURE 6. A comparison of the difference between the maximum and standard levels of oxygen uptake (lower curve) and the speed at which goldfish can swim steadily at various temperatures. The vertical line at 40° C. indicates the ultimate upper incipient lethal temperature of the goldfish.

In Figure 5 it will be seen that while the curve of standard metabolism probably increases with increasing temperature right up to the ultimate incipient lethal temperature (Fry, Brett and Clawson, 1942; Fry, Hart and Walker, 1946), the same is not true of the curve of maximum oxygen uptake. This latter curve reaches a height at about 30° C. which apparently represents the maximum capacity of the system involved, for it is not surpassed at higher temperatures. The consequence of the difference in course of the two curves representing the maximum and standard rates of oxygen uptake is that the difference between them reaches a maximum in the neighborhood of 28° C. and falls off rapidly above 30° C. The difference between the maximum and standard rates is given in the lower panel of Figure 5.

If the difference between the maximum and standard rates of metabolism does approximate the metabolism available for external work, then it would appear that there is an optimum temperature in the neighborhood of 28° C. at which goldfish can perform the most external work. This deduction from the data on metabolism is supported by observations on the relation of temperature to cruising speed (Fry and Hart, 1947). The curve illustrating the relation between temperature and the speed at which goldfish can swim steadily also appears to show a maximum between 20° and 30° C., although somewhat flatter than that for the difference between maximum and standard metabolism. The curves for cruising speed and the metabolic difference are compared in Figure 6. Thus, there seems to be the same correlation between oxygen consumption and activity over the whole temperature range that Spoor (1946) demonstrated at 23–25° C.

Discussion

The data presented on the maximum and standard rates of oxygen uptake point to an explanation of activity-temperature curves which drop at the upper end of the biokinetic range, which is alternative to the theory of thermal destruction usually invoked in such cases. The standard metabolism appears to increase with increasing temperature right up to the ultimate incipient lethal temperature. There is certainly no evidence of destruction or impairment of respiratory enzymes concerned with the standard metabolic rate up to a temperature of 35° C. On the other hand, the curve for maximum metabolism would appear to level off above 30° C. Such levelling off might be caused by a destruction of enzymes at higher temperatures to a degree which produces a balance between the effects of the increased temperature and the ability of the organism to respond, but some proof of this assumption is necessary if it is going to be offered as the explanation. Moreover, to explain the drop in cruising speed above 30° C. on the basis of thermal destruction of enzymes would then require that the explanation be applied to the maximum metabolic rate alone. However, a simpler explanation would be that the levelling of the maximum rate is because the capacity of some part of the organism concerned in oxygen transport is reached at about 30° C. However, whatever may be the explanation of the course of the curve of maximum metabolism, the correlation between activity and metabolism is not with either of the metabolic levels investigated here but with the difference between them. Or more properly, it is with some function of the difference between the two curves which ideally measure the metabolic levels and which our measurements approximate as is indicated by the correlation shown in Figure 6.

With respect to technique it appears that the simplest and most direct methods of obtaining ecologically significant values of oxygen uptake are to measure the maximum rate over a series of varying oxygen tensions down to the asphyxial level, and to measure the standard rate over a range of tensions at which oxygen would not be the limiting factor even for the maximum rate. In the case of the gold fish, for reasons stated above, carbon dioxide tension has not been considered, but for many other species it cannot be so ignored. In many respects however, there seems to be no major objection to combining the effects of carbon dioxide increase and oxygen decrease since they are combined in nature, particularly if the experiments are performed in water low in dissolved minerals so that a maximum increase in carbon dioxide tension will be attained for a given decrease in oxygen. Thus the limiting

values for oxygen attained will approach the maximum that would be expected to be found in nature.

Summary

1. Two levels of oxygen uptake, (1) the lowest point in the resting metabolism in the daily cycle and (2) the maximum steady rate of oxygen uptake found when the fish were stimulated to activity in a rotating chamber, were measured at temperatures from 5° to 35° C.

2. The standard (resting) rate was measured over levels of oxygen high enough to avoid any dependence of the rate on oxygen tension. The maximum rate was measured over a series of oxygen tensions down to the asphyxial level.

3. The standard rate continued to increase with temperature up to 35° C., the highest temperature at which observations were made. The maximum rate was found by interpolation to reach its highest value at about 30° C. and to remain steady or decrease slightly at higher temperatures.

4. The maximum rate of oxygen uptake became dependent upon the oxygen tension between 15 and 40 mm. Hg, depending upon the temperature. These estimates were made in a closed system in which there was an accumulation of the carbon dioxide released in respiration.

5. Oxygen tensions at which the maximum oxygen uptake met only the needs of the standard metabolism were estimated to be between 4 and 25 mm. Hg over the temperature range investigated.

6. At the various temperatures the difference between the maximum and standard metabolic rates is correlated with the rate at which goldfish can swim steadily.

7. It is concluded that the drop in the sustained swimming rate of goldfish at temperatures from 30° to 38° C. is probably due to a decrease in the metabolism available for external work rather than to the thermal destruction of enzymes.

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ADDENDUM

SEMINAR, 5 AUGUST 1947, MARINE BIOLOGICAL LAB., WOODS HOLE, MASS.

An experimental study of the 'second factor' in artificial parthenogenesis in the frog egg. J. R. SHAVER.

Artificial parthenogenetic development of the virgin frog egg into cleavage stages and later embryos requires the introduction of cells or of cellular extracts, the 'second factor,' by pricking the egg with a fine glass needle, or by injection with pipettes at the time of activation of the egg (Bataillon, 1911 a, *C. R. Acad. Sci.*, T. 152, 920–922; Einsele, 1930, *Arch. f. Entwicklungs.*, Bd. 123, 279–300). Bataillon regarded the active principle of the inoculated cells as 'nuclear catalysts,' and Einsele thought the 'second factor' in watery extracts of frog blood and testis was a protein, possibly of enzymatic nature. The experiments here reported show that the injection into virgin eggs of granules and supernatant fluids obtained from homogenates of frog blood, testis, and of certain embryonic stages of the frog, by differential centrifugation, produces parthenogenetic development, the large basophilic granules acting as the 'second factor.' Tissues were extracted in M/200 phosphate buffer, slightly alkaline; granules were obtained by centrifugation of homogenates at 3000–6000 × g, and at 18,000 × g. The usual precautions against sperm contamination were observed. Small granules ('microsomes') obtained at 18,000 × g from blood, testis and frog blastulae gave smaller percentages of parthenogenetic embryos than the larger granules sedimented at 3000–6000 × g from testis and blastulae. Granules from stages earlier than blastulae were ineffective, as were the supernatant fluids from the high-speed centrifugations. Frog serum also produced cleavage when injected, but activity is markedly reduced upon high-speed centrifugation. The majority of parthenogenetic blastulae obtained show uncleaved areas, but the cleaved portions were seen to be nucleated in section. There is evidence that larger granules from tissue homogenates contain succinoxidase, cytochrome oxidase and other enzymes (Brachet and Jeener, *Enzymolog.*, 1944, 13– 196; Chantrenne, *Biochem. et Biophysica Acta*, in press). It is suggested that the second factor may eventually be associated with one or more enzymes present in the granules, rather than with ribonucleic acid itself.